The effects of ovarian enzyme modulators on folliculogenesis and cyst development in the porcine ovary

A thesis submitted by

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for the degree of Doctor of Philosophy

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2007

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To my family:

Dad, Mum and Renu

Abstract

The first aim of the research conducted as part of this thesis was to investigate the expression and activities of 11βHSD enzymes in porcine mural granulosa cells and COCs from small, medium and large antral follicles, as well as from ovarian cysts. Both cloned 11βHSD enzymes (11βHSD1 and 11βHSD2) were expressed in porcine granulosa cells however the results of the expression studies in COCs were inconclusive. In granulosa cells from antral follicles, the 11β-dehydrogenase (11β-DH) activities of the 11βHSD enzymes increased by approximately 3-fold with antral follicle growth ($P<0.01$). Similarly, COCs isolated from large follicles had approximately 10-fold higher 11β-DH activities than COCs from small follicles ($P<0.001$). These results suggested that antral follicle growth occurred alongside increasing levels of intracellular cortisol metabolism in both granulosa cells and COCs. In granulosa cells from ovarian cysts, there were significantly decreased net 11β-DH activities compared to cells from large antral follicles ($P<0.01$), suggesting that decreased cortisol metabolism occurred in the cells of porcine ovarian cysts. The next aim of this thesis was to investigate the levels of ovarian enzyme modulators in FF from small, medium and large antral follicles and in the fluid from ovarian cysts. The levels of inhibitors appeared to decrease in FF with follicle growth but were significantly increased in ovarian cysts ($P<0.01$). These intrafollicular 11βHSD1 inhibitors in FF and cyst fluid were also able to modulate 11βHSD activities in the granulosa cells and COCs. Furthermore, cyst fluid, and the hydrophobic components thereof (which were likely to have included the ovarian enzyme modulators), were shown to significantly increase the rates of porcine oocyte maturation ($P<0.001$). In summary, the findings of the studies conducted in this thesis suggest that the intrafollicular modulators of 11βHSD1 could influence local cortisol-cortisone inter-conversion in granulosa cells and oocytes during antral follicle growth. In addition the ovarian enzyme inhibitors could possibly influence oocyte maturation with follicle growth. In ovarian cysts however, the high levels of intra-follicular 11βHSD1 inhibitors in the cyst fluid, and the decreased levels of intracellular cortisol metabolism observed in the granulosa cells, could be factors contributing to cyst development.

Acknowledgements

Firstly, I would like to acknowledge the financial support I received while doing this PhD, both from the BBSRC and from my CASE sponsor Genus Plc. To my supervisors, Dr. Tony Michael and Dr. Lisa Thurston, you have both given me amazing support and encouragement throughout my years of study with you, and for that I am very grateful. Many thanks to you both for the time you have spent with me teaching me all you can and for the belief you had in me. I am also indebted to Dr. Rachel Webb; chapter 6 would not have been possible without your help and input.

To each of my friends, I am so thankful to have all of you, particularly during the last few months of my study which have undoubtedly been the most difficult. Having said that, I am so glad to have been writing up alongside my fellow final year PhD students, Nana and Abdul. You have provided me so much entertainment and help, and you have really spurred me on to finish my writing. I am glad that your thesis writing has gone really well, you both deserve to go on and do great things after getting your PhDs! To Kim and Vicky, my former lab partners, I am so grateful for your advice and words of encouragements. You have always been there when I needed someone to talk to. I hope that we will always share our love for music festivals and that we keep up the traditions of attending one every year! I would also like to thank my other friends, in and around London, for their patience when I have been at my most busy, I now have time to spend with each and every one of you and that is exactly what I intend to do!

Finally, to my family: I owe you so much for the past few years, you have truly provided me with amazing amounts of encouragement and belief (and financial backing!!). Mum and dad, our chats have really helped me to keep a clear head and ease my stress and Renu, I now have so much more time to spend in the flat with you! During my study time you have always been there with your love and support and I have done this for you as much as for myself. I dedicate this to you.

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Papers

Sunak N, Green DF, Abeydeera LR, Thurston LM and Michael AE (2007) Implication of cortisol and 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes in the development of porcine (*Sus scrofa domestica*) ovarian follicles and cysts. *Reproduction* 133 (6): 1149-1158

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Sunak N, Thurston LM and Michael AE (2004) Effects of ovarian enzyme modulators on folliculogenesis and cyst development in the porcine ovary. *Proceedings of the National Ovarian Workshop (2004)*

Michael AE, Gregory L, Norgate DP, Jonas KC, Chandras C, **Sunak N**, Chin E, Abayasekara DRE, Wood PJ, Cooke BA and Thurston LM (2004) Glucocorticoid metabolism and reproduction: a tale of two enzymes. *Journal of Reproduction Abstract Series 31* Abstract S7

Sunak N, Thurston LM and Michael AE (2004) Changes in ovarian modulators of type 1 11β-hydroxysteroid dehydrogenase (11βHSD1) activity during porcine follicular growth. *Endocrine Abstracts Volume 9* Abstract P79

Sunak N, Thurston LM and Michael AE (2005) Changes in ovarian modulators of type 1 11β-hydroxysteroid dehydrogenase (11βHSD1) activity during porcine ovarian follicle growth. *Journal of Reproduction Abstract Series 32* Abstract O02

Thurston LM, Wilfling L, Sharp V, **Sunak N**, Jonas KC, Prathalingam N and Michael AE (2005) Modulators of type 1 11β-hydroxysteroid dehydrogenase (11βHSD1) isolated from porcine seminal plasma enhance the survival of boar

sperm at room temperature and following cryopreservation. *Journal of Reproduction Abstract Series 32* Abstract O60

Sharp V, Jonas KC, **Sunak N**, Michael AE and Thurston LM (2005) 11β-hydroxysteroid dehydrogenase (11βHSD) activities in the testis and reproductive tract of post-pubertal boars. *Endocrine Abstracts Volume 10* Abstract P66

Sunak N, Sharp V, Wood PJ, Abeydeera LR, Thurston LM and Michael AE (2005) Intra-follicular cortisol:cortisone (F:E) ratios in porcine antral follicles and ovarian cysts; relationship to ovarian modulators of type 1 11β-hydroxysteroid dehydrogenase (11βHSD1) activity. *Endocrine Abstracts Volume 10* Abstract P68

Sunak N, Sharp V, Abeydeera LR, Thurston LM and Michael AE (2006) 11β-hydroxysteroid dehydrogenase (11βHSD) activities in porcine granulosa cells from ovarian follicles and cysts. *Endocrine Abstracts Volume 11* Abstract P717

Sunak N, Abeydeera LR, Thurston LM and Michael AE (2006) Inhibition of cortisol metabolism in porcine granulosa cells by ovarian modulators of type 1 11β-hydroxysteroid dehydrogenase (11βHSD) isolated from porcine large antral follicles and ovarian cysts. *The Endocrine Society 88th Annual Meeting* Abstract P1-399

Sunak N, Abeydeera LR, Thurston LM and Michael AE (2006) Role for the intra-follicular inhibitors of type 1 11β-hydroxysteroid dehydrogenase (11βHSD) activities in the growth of porcine antral follicles and the development of porcine ovarian cysts. *Journal of Reproduction Abstract Series 33* Abstract NO12

Abbreviations

3αHSD	3α-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
11β-DH	11β-dehydrogenase
11βHSD	11β-hydroxysteroid dehydrogenase
11-KSR	11-ketosteroid reductase
17βHSD	17β-hydroxysteroid dehydrogenase
ACRD	apparent cortisone reductase deficiency
ACTH	adrenocorticotrophic hormone
AME	apparent mineralocorticoid excess
AP-1	activator protein-1
APP	acute phase protein
BMP	bone morphogenetic protein
cAMP	cyclic adenosine-3'-5'-monophosphate
CBG	corticosteroid binding protein
CBX	carbenoxolone
CDCA	chenodeoxycholic acid
cDNA	complimentary deoxyribonucleic acid
COC	cumulus-oocyte complex
COD	cystic ovarian disease
CL	corpus luteum
CRH	corticotrophin releasing hormone
Cx	connexin
CYP	cytochrome P450
CYP7A1	7α-hydroxylase
CYP7B1	7β-hydroxylase

CYP11A1	cytochrome P450 side-chain cleavage
CYP11B1	11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17	17α-hydroxylase/17,20 lyase
CYP19	aromatase
CYP21	21α-hydroxylase
CYP26	26-hydroxylase
DHEA	dehydroepiandrosterone
DHEA-S	ehydroepiandrosterone-sulphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DO	denuded oocyte
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FF	follicular fluid
FF-MAS	follicular fluid-meiosis activating sterol
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
G-6-P	glucose-6-phosphate
GA	glycyrrhetinic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GDF	growth differentiation factor
GH	growth hormone
GnRH	gonadotrophin releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GV	germinal vesicle
GVBD	germinal vesicle breakdown
GZ	glycyrrhizic acid
H6PDH	hexose-6-phosphate dehydrogenase
HA	hyaluronic acid
HAS-2	hyaluronic acid synthase-2
HPA	hypothalamo-pituitary-adrenal
HPG	hypothalamo-pituitary-gonadal
IGF-1	insulin-like growth factor-1
IGF-2	insulin-like growth factor-2
IL	interleukin
IVF	*in vitro* fertilisation
IVF-ET	*n vitro* fertilisation-embryo transfer
IVM	*in vitro* maturation
K^+	potassium ions
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
LH	luteinising hormone
LHR	luteinising hormone receptor
MII	metaphase II

MAO	morpholino anti-sense oligonucleotide
$MgCl_2$	magnesium chloride
M-MLV	Moloney Murine Leukemia Virus
MPF	maturation promoting factor
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MW	molecular weight
Na^+	sodium ions
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
$NaH_2PO_4.2H_2O$	sodium di-hydrogen orthophosphate dihydrate
$Na_2HPO_4.12H_2O$	di-sodium hydrogen orthophosphate 12-hydrate
NFκ-B	nuclear factor κ-B
OD	optical density
PBS	phosphate-buffered saline
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PGHS-2	prostaglandin H synthase-2
PI	propidium iodide
PKA	protein kinase A
PR	progesterone receptor
RIA	radioimmunoassay
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction

SDR	short-chain dehydrogenase/reductase
SER	smooth endoplasmic reticulum
SHBG	sex-hormone-binding globulin
siRNA	small interfering ribonucleic acid
StAR	*st*eroidogenic *a*cute *r*egulatory protein
TF	transcription factor
TGF-β	transforming growth factor-β
TLC	thin-layer chromatography
TNFα	tumour necrosis factor-α
TRITC	tetramethylrhodamine isothiocyanate
v/v	volume per volume
w/v	weight per volume
ZF	zona fasciculata
ZG	zona glomerulosa
ZP	zona pellucida
ZR	zona reticularis

Chapter 1

General Introduction

1.1 The Ovary

In the female pig, fully differentiated ovaries are visible at 31-32 days *post coitus* (Allen, 1904). Around 62 days after conception (Mauleon, 1964), during the mid-gestational period (Allen, 1904), primordial follicles are formed, each comprised of a single layer of flattened granulosa cells surrounding an immature germ cell. At birth, there are approximately 500,000 primordial follicles in the porcine ovary (Black and Erickson, 1968), supplying enough gametes to span a female's reproductive life. At puberty, between 4 and 7 months of age in the pig, the number of porcine primordial follicles decreases to 420,000 (Gosden and Telfer, 1987). A summary of oogenesis and folliculogenesis will be given in sections 1.1.1 and 1.1.2, respectively.

1.1.1 Overview of oogenesis

Between days 20-50 of embryonic development, the number of germ cells in the pig embryo increases from 5,000 to 110,000: the peak number of gametes (see review by Hunter (2000)). The immature germ cells initially differentiate into oogonia then divide by mitosis to form primary oocytes. When mitotic divisions cease, each oocyte enters its first meiotic division. Meiosis in oocytes arrests at two stages, the first of which is the prophase of the first meiotic division, also known as the germinal vesicle (GV) stage. The GV refers to the nucleus, which, at this time, has swelled with fluid and contains condensed chromosomes. By day 35 after birth, all oocytes are arrested in the first meiotic prophase (Black and Erickson, 1968). Meiosis does not resume from the first prophase until the oocyte responds to the cyclic surge of gonadotrophins associated with the ovarian cycle (described in section 1.2.2) and in the pig, meiosis can only resume in oocytes from antral follicles of 0.8-1.6mm in diameter (Motlik *et al.*, 1984; Hunter, 2000). After the resumption of meiosis from the first prophase stage, the oocyte progresses through the remaining stages of the metaphase stage of the first meiotic division, during which GV breakdown (GVBD) occurs. At the end of the first meiotic division, the first polar body is extruded and meiosis continues into the the second meiotic division until the next point of arrest at the second metaphase (MII) stage.

1.1.2 The porcine oestrous cycle

One complete porcine oestrous cycle takes 21 days, compared to 28 days which is the duration of one ovarian cycle in women. This is due to a shorter follicular phase of 7 days in the pig, rather than 14 days in women. The porcine oestrous cycle can be split into three phases: proestrus (follicular phase), oestrus (sexual receptivity) and diestrus (luteal phase; Figure 1.1). In the proestrus phase, during which time oestradiol output is high, immature follicles develop into ovulatory (large antral) follicles. Towards the end of the proestrus phase, levels of oestradiol and the gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH), rise. This surge of LH coincides with the onset of oestrus and induction of ovulation. The porcine cycle begins on the first day of oestrus, after which a female pig is in oestrus for 1-3 days. Once ovulation has occurred, the ruptured follicles are remodelled to form a corpus luteum (CL). LH, FSH and oestradiol concentrations decrease and progesterone levels rise during the luteal phase.

Species such as humans, cows and sheep exhibit "waves" of follicular growth and selection however, pig follicles show continuous growth and atresia, with a new cohort developing during each luteal phase. This may occur as pigs are polyovulatory and can develop 15-20 ovulatory follicles, possibly due to that fact porcine gonadotrophins remain at sufficient levels to maintain follicle growth. The approximate period of development from a primordial to an ovulatory follicle is 6 months in the sheep and cow (Campbell *et al.*, 2003) but 4 months in the pig (Morbeck *et al.*, 1992). A diagram depicting the stages of folliculogenesis is given in Figure 1.2 and the process of folliculogenesis is discussed next.

1.1.2.1 Primordial follicles become preantral follicles

Porcine primordial follicles grow to 150-300μm in diameter at the preantral stage (Telfer *et al.*, 2000), during which time, granulosa cells proliferate in the follicle wall, while the porcine oocyte also grows to 30μm in diameter (Morbeck *et al.*, 1992). During preantral follicle formation, the oocyte can synthesise and translate

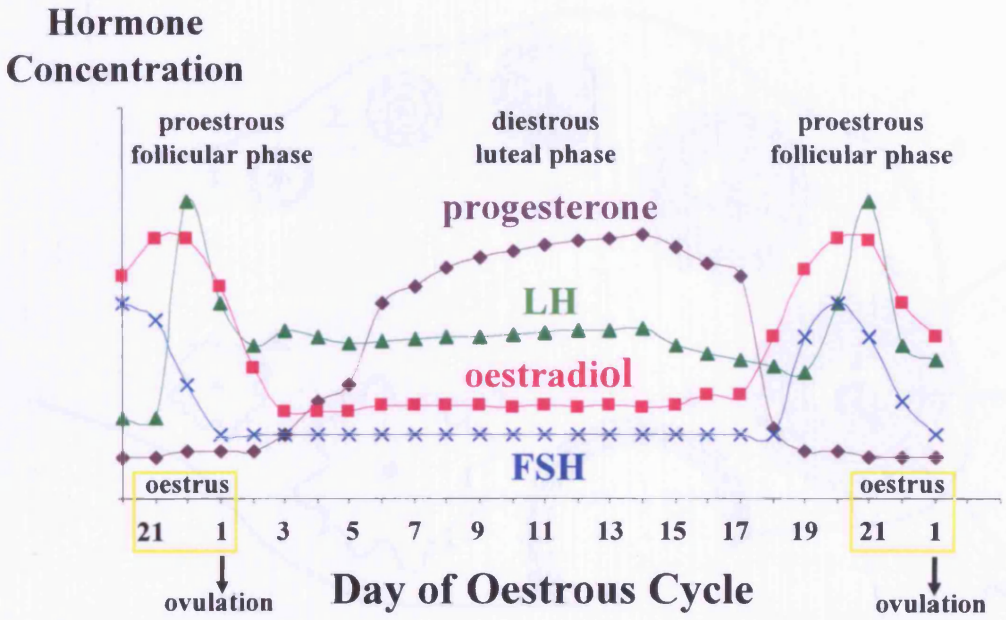


Figure 1.1. The relative concentrations of progesterone, luteinising hormone (LH), oestradiol and follicle-stimulating hormone (FSH) in the systemic plasma during the proestrus, oestrus and diestrus phases of the 21-day porcine oestrous cycle. Progesterone concentrations are represented by purple diamonds, LH by green triangles, oestradiol by pink squares and FSH by blue crosses. Oestrus is shown to occur between days 21 and 1 of the porcine oestrous cycle in this figure, with ovulation occurring at day 1. The figure was modified from Senger (2005).

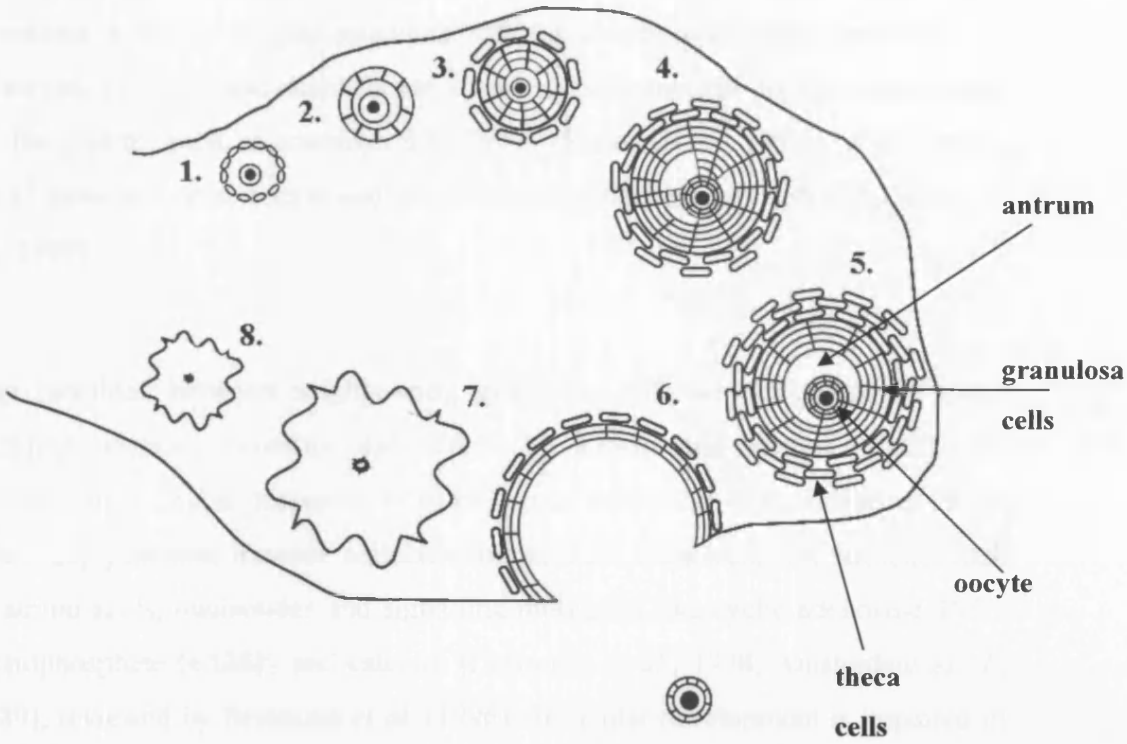


Figure 1.2. An overview of the stages of folliculogenesis in the porcine ovary. From the top left the stages of growth are as follows: (1) primordial follicle, (2) early primary follicle, (3) late primary follicle, (4) preantral follicle, (5) antral follicle, (6) ovulation (showing follicle rupture and oocyte release), (7) corpus lutem and (8) regressing corpus lutem. In the illustration of the antral follicle (5) the locations of the antrum, the granulosa and theca cells and the oocyte are indicated by the solid black arrows. The figure was modified from Senger (2005).

messenger ribonucleic acid (mRNA) into proteins, such as zona pellucida-1, -2 and -3 (ZP-1, -2 and -3) (reviewed by Wasserman (1996)). These proteins aggregate to form a thick, translucent layer called the zona pellucida (ZP), separating the oocyte and granulosa cells. Transzonal projections extend from the granulosa cells to form gap junctions with the oocyte membrane (Anderson and Albertini, 1976). These channels are composed of connexin proteins synthesised by the oocyte, such as connexin 37 (Cx37) (Simon *et al.*, 1997). Mice lacking Cx37 have abnormal oocyte and follicle development and are infertile (Simon *et al.*, 1997).

Gap junctions between neighbouring granulosa cells are comprised of many different connexin proteins (see review by Kidder and Mhwai (2002), one example of which is connexin 43 (Cx43) (see review by Grazul-Bilska (1997). These gap junctions increase cellular communication and transport nutrients such as amino acids, nucleotides and signalling molecules like cyclic adenosine-3'-5'-monophosphate (cAMP) and calcium (Lawrence *et al.*, 1978; Amsterdam *et al.*, 1989), reviewed by Bruzzone *et al.* (1996). Follicular development is impaired in Cx43-deficient mice at the primary stage (Juneja *et al.*, 1999). The expression of Cx26, Cx32 (Kwiatkowski *et al.*, 1994) and Cx43 (Sasson and Amsterdam, 2002) can be increased by the glucocorticoid hormones, which are a focus of this thesis.

Local growth factors, such as epidermal growth factor (EGF) (Gospodarowicz and Bialecki, 1978; Hammond and English, 1987) and insulin-like growth factor (IGF)-1 and -2 (reviewed by Adashi *et al.* (1985)) (Kolodziejczyk *et al.*, 2001) have been shown to synergistically increase porcine granulosa cell proliferation. The oocyte produces growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15, also known as GDF9B), which can also stimulate proliferation of cultured mouse and rat granulosa cells (Matzuk, 2000; Otsuka *et al.*, 2000; Vitt *et al.*, 2000). The GDF and BMP proteins are two members of the transforming growth factor-β (TGF-β) family (reviewed by Elvin *et al.* (2000) and Erickson and Shimasaki (2000)). Additionally, BMP-6 has been reported to stimulate porcine granulosa cell proliferation (Brankin *et al.*, 2005)

and GDF-9 could induce human preantral follicle growth (Hreinsson *et al.*, 2002). Follicle growth is arrested in mice and sheep lacking GDF-9 and BMP-15 (Chang *et al.*, 2002), indicating the need for these oocyte-derived proteins in preantral follicle formation. GDF-9 may also stimulate cumulus expansion and prevent apoptosis, discussed in sections 1.2.2 and 1.2.3, respectively. Alhough the oocyte is transcriptionally active, it is arrested in the first meiotic prophase at this time and meiosis cannot resume until the follicle acquires an antrum and falls under the control of FSH and LH. At the preantral stage the follicle wall also acquires a theca cell layer, derived from the differentiation of ovarian stromal cells aggregated around the granulosa cells (reviewed by Erickson *et al.* (1985)). The theca and granulosa cells are distinct and separated by a basement membrane called the basal lamina.

1.1.2.2 Preantral follicles develop into ovulatory follicles

Cell proliferation in the preantral follicle wall continues to increase follicle diameter. The granulosa cells differentiate into four distinct layers: the corona radiata (around the oocyte), cumulus oophorus, periantral and mural granulosa cell layer (adjacent to the basal lamina). The cumulus region is a 'stalk' of condensed cells linking the corona radiata and periantral granulosa cells to form a cumulus-oocyte complex (COC). The theca cells differentiate into the theca externa and interna. The theca externa contains fibrous, connective tissue which encloses and supports the follicle (Erickson *et al.*, 1985). The theca interna contains interstitial cells which become vascularised to form a blood capillary network that delivers nutrients and hormones to the follicle and removes secreted factors and waste products. The theca interna and mural granulosa cells acquire receptors for LH and FSH, respectively. This will be discussed further in section 1.2.

Whilst cellular proliferation occurs, spaces appear in the follicle which aggregate to form one cavity called the antrum. Porcine antral follicles can first be detected 62-90 days after birth (Mauleon, 1964; Oxender *et al.*, 1979). Paracrine factors shown to induce antrum formation in mice are granulosa-derived kit ligand

(Yoshida *et al.*, 1997) and Cx37 (Simon *et al.*, 1997), as without either protein, no antral follicles develop and a female is infertile. The antrum fills with follicular fluid (FF) composed serum transudate and oocyte and granulosa cell secretions, such as mucopolysaccharides (see review by Edwards (1974)). FF also contains beta-carotene and bilirubin, two pigments that absorb at 455nm in the visible spectrum. Of the two, bilirubin is the primary compound that gives FF its yellow colour (Bayer *et al.*, 1992).

Antral follicles increase in diameter due to granulosa and theca cell proliferation, and through antrum expansion with the accumulation of FF. Once a porcine antral follicle reaches 6mm in diameter it is selected to become an ovulatory follicle (Dalin, 1987). In a porcine preovulatory follicle, the oocyte is approximately 120μm in diameter (Hunter, 2000). Ovulation will only occur when the follicle is stimulated by the gonadotrophins, which will be discussed next. The follicles that are not selected to ovulate regress through atresia, which will be described further in section 1.2.3.

1.2 The Endocrine Regulation of Folliculogenesis

The development of an antral follicle is determined by LH and FSH, through their receptors (LHRs and FSHRs) which are initially expressed on the theca interna and the granulosa cells in the follicle wall, respectively in the primary follicles. Angiogenesis in the follicle aids the access of circulating gonadotrophins. These hormones are released in a pulsatile fashion by the anterior pituitary gland in response to pulsatile gonadotrophin releasing hormone (GnRH) secretion from the hypothalamus. GnRH production is, in turn, regulated by oestradiol. The importance of the gonadotrophins is indicated by the fact that, on removal of the pituitary gland, follicles will not develop beyond the early antral stage. The actions of the gonadotrophins can also prevent follicle atresia (Chun *et al.*, 1996), a process described in section 1.2.3.

FSH is important for early antral follicle growth. The highest levels of FSHR mRNA were found in mural granulosa cells from small porcine antral follicles (Slomczynska *et al.*, 2001). FSH stimulates production of oestradiol during the follicular phase, and induces the expression of LHRs (Erickson *et al.*, 1979) and steroidogenic enzymes such as CYP19 (aromatase). FSH was also shown to increase granulosa cell proliferation in women (Gougeon and Testart, 1990) by upregulating the expression of cyclin D2, a cell-cycle protein (Roy and Greenwald, 1989). While FSH is critical to early antral follicle recruitment, LH becomes the more important gonadotrophin as a follicle approaches ovulation (Zeleznik and Hillier, 1984) (see review by Hillier (2001)). The ovulation of an large antral follicle is associated with an increase in LH pulse frequency (Stock and Fortune, 1993). The events of ovulation will be described in section 1.2.2.

1.2.1 The "two-cell-two-gonadotrophin" model

The theca cells express the enzymes CYP11A1 (cytochrome P450 cholesterol side-chain cleavage), 3β-hydroxysteroid dehydrogenase (3βHSD) and CYP17 (17α-hydroxylase/17,20 lyase), which are involved in the synthesis of androstenedione from cholesterol (via the reaction steps described in section 1.3.2 and depicted in Figure 1.4). The binding of LH to LHRs on the theca cells upregulates the expression of these steroidogenic enzymes, via an increase in cAMP production. The granulosa cells do not express CYP17, as demonstrated in the porcine ovary by Shores and Hunter (1999) such that the theca cells have to provide the granulosa cells with androgen substrate. Under the influence of FSH, CYP19 expression in the granulosa cells is upregulated to increase the conversion of androstenedione to oestrone, which, in turn, is reduced to oestradiol by 17β-hydroxysteroid dehydrogenase (17βHSD). This is the "two-cell-two-gonadotrophin" model of follicular oestradiol biosynthesis (Figure 1.3) (Falck, 1959; Moon *et al.*, 1975; Armstrong and Papkoff, 1976).

Theca cells also produce progesterone after LH stimulation (Bogovich *et al.*, 1986), presumably through the upregulation of CYP11A1 and 3βHSD by LH, as

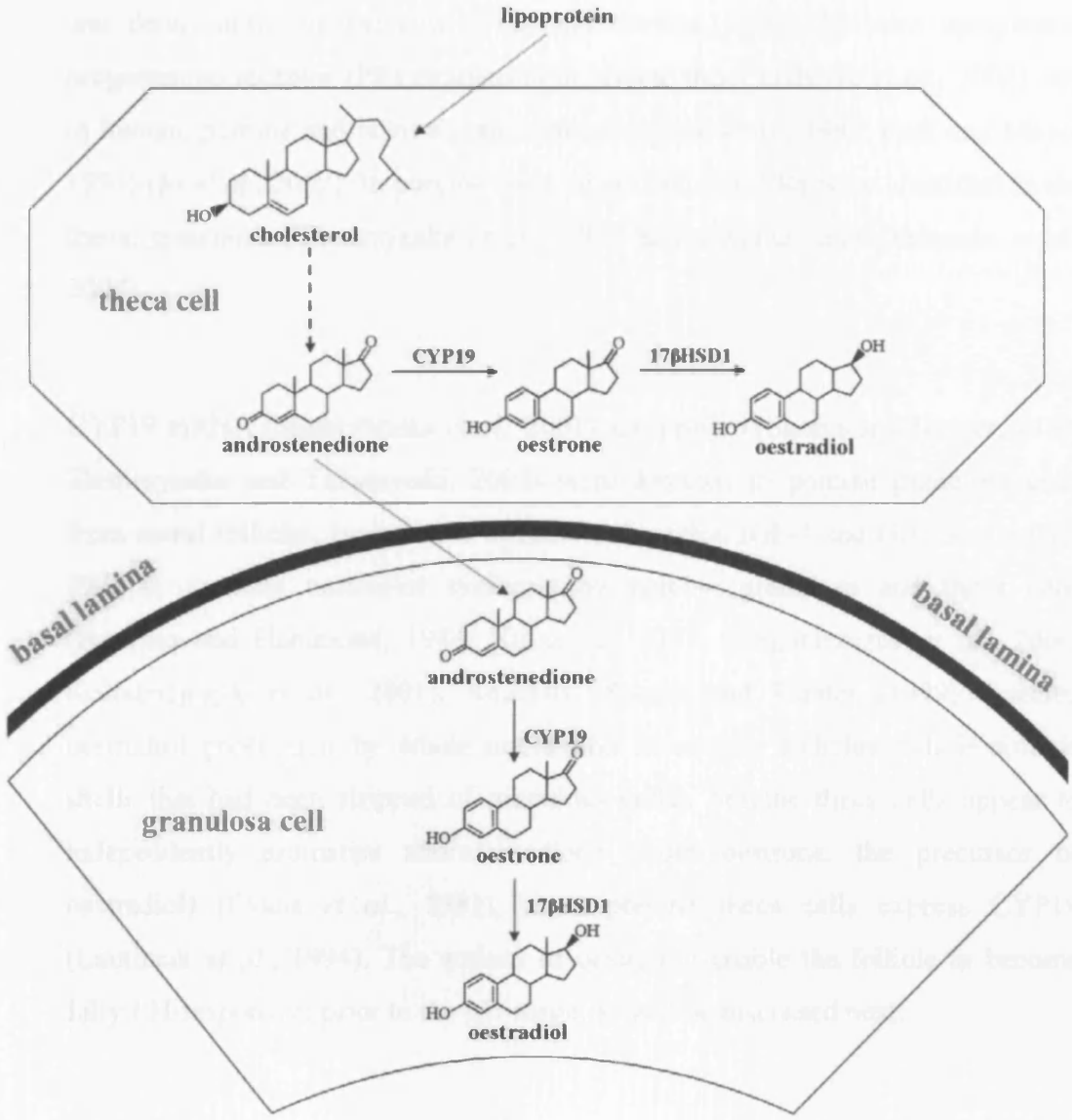


Figure 1.3. An illustration of the "two-cell-two-gonadotrophin" model showing steroidogenesis in the theca and granulosa cells of the porcine ovarian follicle. Arrows with dotted black lines demonstrate the direction of movement of hormones. The single arrow with the dashed black line in the theca cell represents the reactions of steroidogenic pathway from cholesterol to androstenedione (these steps are fully illustrated in Figure 1.4). In the image of each cell type, arrows with solid black lines indicate direct steroid conversions; the enzymes that catalyse these reactions are shown in blue uppercase font. The figure was modified from Brook and Marshall (2001).

was demonstrated in the cow (Voss and Fortune, 1993). LH also upregulates progesterone receptor (PR) expression in bovine theca cells (Jo *et al.*, 2002) and in human, porcine and bovine granulosa cells (Iwai *et al.*, 1991; Park and Mayo, 1991) (Jo *et al.*, 2002). In porcine large antral follicles, PRs were identified in the theca, granulosa (Slomczynska *et al.*, 2000) and cumulus cells (Shimada *et al.*, 2004).

CYP19 mRNA (Slomczynska *et al.*, 2001) and protein (Shores and Hunter, 1999; Slomczynska and Tabarowski, 2001) were detected in porcine granulosa cells from antral follicles, irrespective of follicle diameter. IGF-1 and GH can act with FSH to increase oestradiol synthesis by porcine granulosa and theca cells (Baranao and Hammond, 1984; Xu *et al.*, 1997; Gregoraszczuk *et al.*, 2000; Kolodziejczyk *et al.*, 2001). Recently, Shores and Hunter (1999) detected oestradiol production by whole antral and theca-only follicles (whole follicle shells that had been stripped of granulosa cells). Porcine theca cells appear to independently aromatise androstenedione (from oestrone, the precursor of oestradiol) (Evans *et al.*, 1981), since porcine theca cells express CYP19 (Lautincik *et al.*, 1994). The actions of oestradiol enable the follicle to become fully LH-responsive prior to the LH surge, as will be discussed next.

1.2.2 Oocyte maturation and the role of LH

Once a follicle reaches dominance, all FSH-induced genes can be stimulated by LH, increasing the expression of genes encoding the enzymes for oestradiol production (Zeleznik and Hillier, 1984; Hillier, 2001). Oestradiol mediates FSH-stimulated LHR expression in theca and granulosa cells (see review by Richards (1994)) (Robker *et al.*, 2000), amplifying the responsiveness of the follicle to LH. LH can induce follicle vascularisation as a means of receiving further hormone stimulation, through a positive feedback effect. LH was shown to upregulate the expression of vascular endothelial growth factor (VEGF), which directs the production of new blood vessels in endothelial cells (Ravindranath *et al.*, 1992; Hillier, 2001). The extensive vascularisation of an ovulatory follicle is one of the

factors that distinguishes it from an atretic follicle (see review by Richards (1980)) (Maxson *et al.*, 1985; Jiang *et al.*, 2003).

Oestradiol can exert a negative feedback effect on GnRH production in the hypothalamus, therefore also limiting gonadotrophin secretion from the anterior pituitary. Physiologically, low levels of oestradiol can suppress GnRH production, but prior to the LH surge, oestradiol output increases by 2-fold. At this time, high oestradiol levels can exert a positive feedback effect on GnRH levels. Gonadotrophin secretion from the anterior pituitary is increased as a result, however, the production of inhibin A by the granulosa cells can inhibit FSH release (Johnson and Everitt, 1999), therefore preovulatory follicles only experience a surge in LH.

In the COC, the LH surge upregulates the expression of prostaglandin H synthase-2 (PGHS-2) (Morris and Richards, 1993), allowing the cumulus cells to synthesise prostaglandins. PGHS-2 knockout mice are infertile and do not ovulate (Lim *et al.*, 1997) though these mice appear to demonstrate normal folliculogenesis (Dinchuk *et al.*, 1995). This raises the possibility that PGHS-2 knockout mice suffer defects in cumulus expansion that may prevent ovulation occurring (Davis *et al.*, 1999). Recently prostaglandin E2 has been shown to upregulate the synthesis of EGF-like factors (Ben-Ami *et al.*, 2006). The EGF-like factors are thought to be involved in cumulus expansion and oocyte maturation; this will be discussed later in this section. LH also increases hyaluronan synthase-2 (HAS-2) expression, enabling cumulus cells to produce hyaluronic acid (HA), which binds proteoglycans to form an extracellular matrix between the cumulus cells (Salustri *et al.*, 1989). When HA becomes hydrated, the cumulus cells separate and become embedded in a sticky, mucified matrix (reviewed by Eppig (2001)). This process is cumulus expansion and involves the breakdown of the Cx37-rich gap junctions between the cumulus cells and the oocyte. The corona radiata cells still maintain contact with the oocyte, suggesting that oocyte maturation does require some cellular communication (Mattioli *et al.*, 1998).

The loss of gap junctions within the COC during cumulus expansion induces the resumption of meiosis in the preovulatory oocyte. Before this stage, meiotic arrest at the first prophase appears to be upheld by cAMP in the oocyte (Magnusson and Hillensjo, 1977; Racowsky, 1985), though the source of cAMP production is unknown. It is generally accepted that the cAMP passes from other follicle cells to the oocyte through gap junctions (Anderson and Albertini, 1976) Thus cumulus expansion appears to liberate the oocyte from high cAMP levels as the breakdown of gap junction communication between the oocyte and the follicular cells prevents cAMP reaching the oocyte (Dekel *et al.*, 1981). Carbenoxolone (CBX) is a synthetic derivative of glycyrrhetinic acid (GA; see section 1.4.5) which appears to block gap junctions; a result that presumably mimics the loss of contacts between the oocyte and cumulus cells during cumulus expansion. Incubation of rat ovarian follicles with CBX induced oocyte maturation in rat oocytes while decreasing concentrations of intrcellular cAMP in the oocyte (Sela-Abramovich *et al.*, 2006).

The drop in cAMP levels after cumulus expansion indirectly activates maturation promoting factor (MPF) (Masui and Market, 1971). MPF is a complex of cyclin-dependent kinase (CDK-1, also known as p34cdc2) and cyclin B1 (Gautier *et al.*, 1990; de Vantery *et al.*, 1997; Kanatsu-Shinohara *et al.*, 2000). MPF resumes meiosis in the oocyte, from its arrest at the first meiotic prophase up to the next point of arrest at the second metaphase (MII), as described in section 1.1.1. The decline in cAMP concentrations within the oocyte enables the phosphatase CDC25 to dephosphorylate CDK-1 and so activate MPF. It appears that murine oocytes lacking the *Cdc25b* gene encoding CDC25 cannot resume meiosis (Lincoln *et al.*, 2002). During meiotic arrest, high levels of intracellular cAMP in the oocyte activates protein kinase A (PKA), inducing WEE1/MYT1 kinase activity (see review by Mehlmann (2005)). This kinase phosphorylates CDK-1 at threonine 14 and tyrosine 15, to render MPF inactive until such a time as cdc25 can dephosphorylate CDK-1 (Gould and Nurse, 1989).

Paracrine factors produced within the ovary are also important in modulating oocyte maturation after the LH surge. Gonadotrophin-treatment appears to upregulate EGF-like factors (amphiregulin, epiregulin, and beta-cellulin) in mouse and rat granulosa cells (Park *et al.*, 2004; Sekiguchi *et al.*, 2004). These proteins have been shown to increase GVBD in mouse oocytes, cause cumulus expansion in porcine COCs and increase the number of porcine oocytes which resumed meiosis (Sirotkin *et al.*, 2000). Furthermore, inhibiting the EGF receptor in cultured mouse follicles can block LH-induced oocyte maturation (Park *et al.*, 2004). The oocyte itself is also thought to secrete an unknown factor to mediate cumulus expansion (see review by Salustri *et al.* (1996)). GDF-9 is a candidate for this factor as it can induce the *in vitro* expansion of cultured mouse cumulus cells and could also increase HAS-2 and PGHS2 mRNA expression in mouse granulosa cells (Elvin *et al.*, 1999).

When meiosis resumes in the oocyte, GVBD begins through the fragmentation of the membrane enclosing the chromosomes. The first meiotic division yields one cell with the majority of the cytoplasm and half of the chromosomes, this is the secondary oocyte. The other "cell" is the polar body, comprised of the remaining chromosomes and little cytoplasm. This is discarded in a process known as extrusion whereby the oocyte loses redundant genetic material. The chromosomes in the secondary oocyte now enter the second meiotic division but arrest at the MII stage. Only during fertilisation, when the MII oocyte fuses with a spermatozoon, does the completion of oocyte meiosis occur.

1.2.3 Follicular atresia

Once a porcine antral follicle reaches 6mm in diameter it is destined for ovulation (Dalin, 1987). A morphologically healthy follicle has a well-vascularised wall and a translucent antrum with no free-floating particles (Maxson *et al.*, 1985; Guthrie *et al.*, 1995). At any stage of early folliculogenesis, however, a follicle may become atretic and degenerate (Byskov, 1978; Yu *et al.*, 2004). In terms of intrafollicular hormone concentrations in healthy versus atretic porcine antral follicles, oestradiol concentrations were, on average, 123±50nM in healthy

follicles and only 18±5nM in atretic follicles (Maxson *et al.*, 1985). Healthy porcine preovulatory follicles had a progesterone content of between 219 and 1945nM (Conley *et al.*, 1994).

A follicle can become atretic when it does not develop correctly, is not selected for ovulation or does not experience the correct ratio of FSH, LH and oestradiol. The occurrence of apoptosis in the granulosa cells is thought to induce follicular atresia. Apoptosis involves the activation of a downstream cascade of caspase enzymes. Caspase-3, caspase-9 and *a*poptotic *p*rotease-*a*ctivating *f*actor 1 (Apaf1) mRNAs were detected in granulosa cells from porcine atretic follicles (Matsui *et al.*, 2003; Berardinelli *et al.*, 2004). As well as inducing granulosa cell proliferation and cumulus expansion, GDF-9 may be anti-apoptotic. In large preantral and small antral rat follicles, GDF-9 appeared to inhibit granulosa cell death (Orisaka *et al.*, 2006). As mentioned above, FSH, IGF-1 and EGF were shown to inhibit apoptosis in cultured rat antral follicles *in vitro* (Chun *et al.*, 1996), and FSH and IGF-1 also had anti-apoptotic effects in porcine granulosa cells *in vitro* (Guthrie *et al.*, 1998). Glucocorticoids are also thought to protect granulosa cells from apoptosis; this will be discussed further in section 1.3.3.

1.2.4 Abnormalities of folliculogenesis – ovarian cyst development

In domestic animals, such as pigs and cows, external factors have been associated with cystic ovarian disease (COD), including season, nutrition, milk production and puerperal stress (reviewed by Vanholder *et al.* (2006)). The underlying mechanisms behind COD are yet to be fully elucidated. If an ovulatory follicle in the porcine ovary becomes anovulatory, a spontaneous ovarian cyst of over 20mm in diameter may form.

COD in domestic animals and PCOS in women may be induced by stress, which is thought to activate the hypothalamo-pituitary-adrenal (HPA) axis (Kamel and Kubajak, 1987). Stress stimulates corticotrophin releasing hormone (CRH) secretion from the hypothalamus which induces adrenocorticotrophic hormone

(ACTH) production from the anterior pituitary, in turn, increasing glucocorticoid production in the adrenal glands. More details of the HPA axis will be discussed in section 1.3.2. An increase in CRH release was shown to suppress the activity of the hypothalamic GnRH pulse generator which decreases GnRH and LH release (Williams *et al.*, 1990). Furthermore, increased concentrations of ACTH appeared to cause a decreased LH pulse frequency in heifers (Dobson *et al.*, 1988; Williams *et al.*, 1990; Dobson *et al.*, 2000; Ribadu *et al.*, 2000). In pigs, elevated glucocorticoid production inhibited GnRH and LH release (Scholten and Liptrap, 1978). Therefore, spontaneous ovarian cysts such as those seen in porcine and bovine COD could be attributed to a decreased LH pulse frequency. Hence COD may be associated with alterations in the HPA and the hypothalamo-pituitary-gonadal (HPG) axis.

An additional hypothesis is that stress perpetuates COD by increasing the sympathetic innervation of the ovary (Schenker *et al.*, 1992). Glucocorticoids were shown to increase the activity of sympathetic intraovarian nerves in pigs (Jana *et al.*, 2005) humans (Nakamura, 1990) and rats (Paredes *et al.*, 1998). In the pig, treatment of cystic ovaries with the synthetic glucocorticoid dexamethasone increased progesterone concentrations, but decreased levels of androgens and oestrogens in the cyst fluid and wall, compared to large antral follicles. Therefore, glucocorticoids may mediate cyst formation through activating the sympathetic nervous system in the ovary, possibly accompanied by altered ovarian steroidogenesis. In the cow, oestradiol, progesterone and androstenedione concentrations were higher in cysts than ovulatory follicles (Calder *et al.*, 2001). Therefore, a modification of follicular hormone concentrations in porcine or bovine ovarian cysts compared to antral follicles may be an indication of COD.

1.3 The Adrenal Glands and HPA Axis

1.3.1 Morphology of the adrenal glands and steroidogenesis

Each adrenal gland comprises an inner medulla and an outer cortex. The adrenal cortex contains three distinct cellular regions, the outermost of which is the zona glomerulosa (ZG), then the zona fasciculata (ZF) and the zona reticularis (ZR), nearest to the medulla. Each zone is able to synthesise steroid hormones, which are all derived from cholesterol (Figure 1.4). The cells in the outermost region of the adrenal cortex, the ZG, express CYP11A1 3βHSD, CYP17, CYP21 (21α-hydroxylase) and CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase). Therefore, the final product of the ZG is the mineralocorticoid, aldosterone. This hormone can bind intracellular mineralocorticoid receptors (MRs) in the distal convoluted tubule and collecting ducts of the kidney nephrons, to regulate the reabsorption of sodium ions (Na^+) and water, and the loss of potassium ions (K^+), in the urine. The ZF expresses CYP11A1, 3βHSD, CYP21 and CYP11B1 therefore, the cells in the central region of the adrenal cortex can produce the glucocorticoid cortisol. The cells of the innermost region of the adrenal cortex, the ZR, express CYP17 but low amounts of 3βHSD (Endoh *et al.*, 1996). Thus the ZR synthesises the "adrenal androgens", dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEA-S).

1.3.2 Production of cortisol by the zona fasciculata

Cortisol is the predominant glucocorticoid produced by the majority of mammals. Corticosterone is the principal glucocorticoid for a small group of mammals, such as mice, rats and rabbits but in most mammals, corticosterone is simply a steroid intermediate that is converted to aldosterone. Since this thesis is focused on aspects of porcine ovarian function, cortisol will be the focus of discussion.

As mentioned in section 1.2.4, during chronic stress, there is an increase in CRH and ACTH release from the hypothalamus and anterior pituitary, respectively. ACTH can stimulate cortisol production by upregulating the expression of enzymes in the steroidogenic pathway (reviewed by Sewer and Waterman

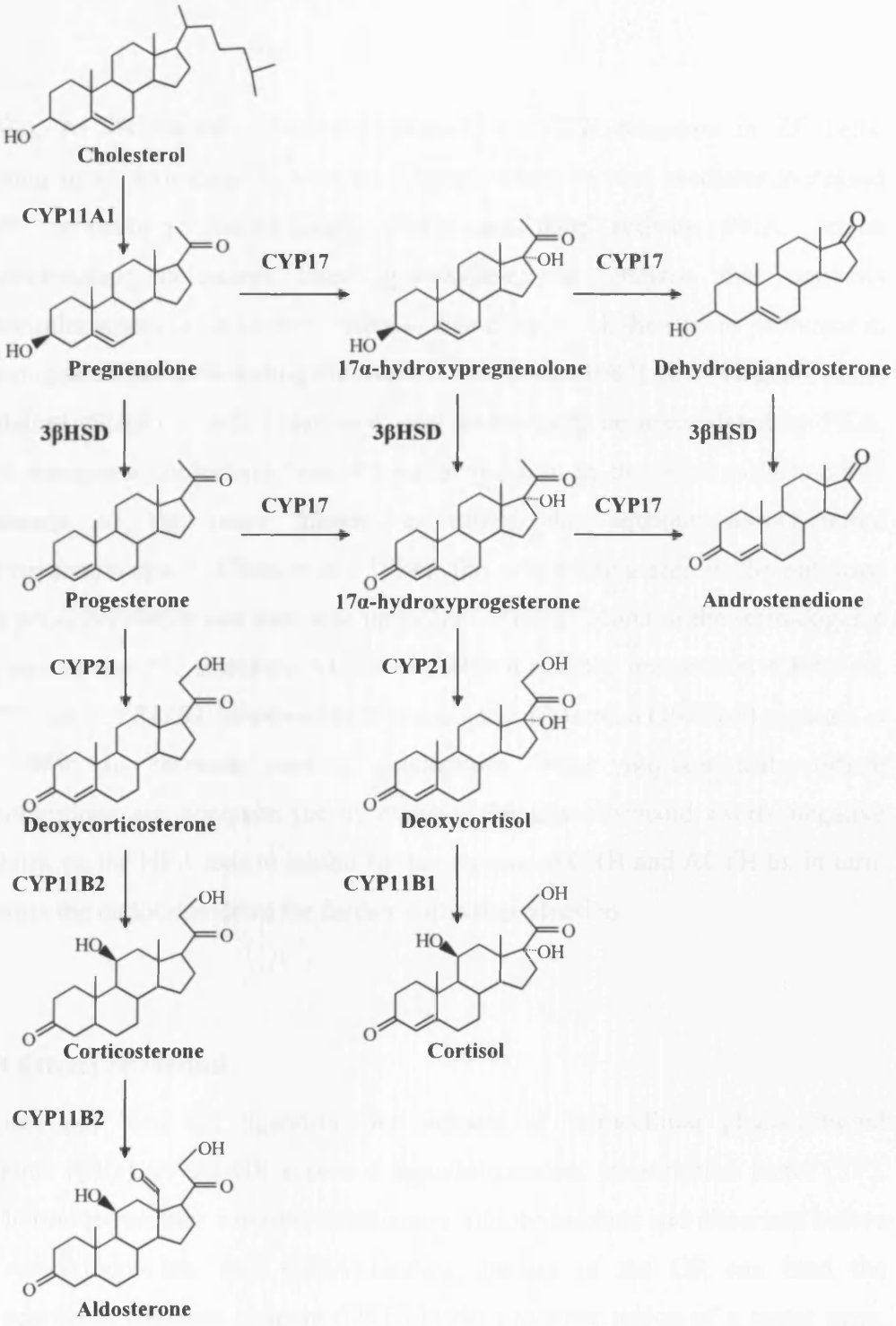


Figure 1.4. The steps of the steroidogenic pathway that occur in the adrenal glands. All steroids are labelled, with the arrows indicating direct conversions between steroids. The enzymes that catalyse the reactions are shown in uppercase font. Within steroid structures, all α-bonds are represented by dotted lines and β-bonds are illustrated as solid black triangles. This figure was modified from (Brook and Marshall, 2001).

(2003)). ACTH initially binds to cell-surface ACTH receptors in ZF cells, resulting in an activation of adenylyl cyclase, which in turn, mediates increased cAMP production. Intracellular cAMP can then activate PKA, which phosphorylates cholesteryl ester hydroxylase, the enzyme that converts intracellular stores of cholesteryl esters to free cholesterol, the steroid precursor in steroidogenic tissues, including the ovary (Holm *et al.*, 1987). *St*eroidogenic *a*cute *r*egulatory (StAR) protein expression may additionally be upregulated by PKA. StAR transports cholesterol from its initial location in the outer mitochondrial membrane to the inner membrane, across the aqueous mitochondrial intermembrane space (Clark *et al.*, 1994): the rate-limiting step in the pathway. PKA phosphorylation can then also upregulate the expression of the steroidogenic enzymes in the ZF, therefore ACTH can also indirectly upregulates *CYP11A1*, *CYP21*, and *CYP11B1* (reviewed by Simpson and Waterman (1988) (Boggaram *et al.*, 1989) to increase cortisol production. When physiological cortisol concentrations are adequate (or in excess), the glucocorticoid exerts negative feedback on the HPA axis to inhibit further release of CRH and ACTH to, in turn, suppress the endocrine drive for further cortisol production.

1.3.3 Effects of cortisol

Cortisol can bind the ligand-binding domain of intracellular glucocorticoid receptors (GRs), so the GR acts as a ligand-dependent transcription factor (TF). The hormone-receptor complex translocates into the nucleus and dimerises before the deoxyribonucleic acid (DNA)-binding domain of the GR can bind the glucocorticoid response element (GRE) in the promoter region of a target gene. The binding of the GR to the GRE can increase or decrease gene transcription.

The glucocorticoids are so-called because they can elevate the plasma concentrations of glucose. In the muscle, adipose and lymphoid tissue, cortisol induces a breakdown of glycogen, however, in the liver, cortisol stimulates glycogen synthesis and storage, and gluconeogenesis (Brook and Marshall, 2001). At the same time all other tissues decrease their use of glucose so, along with the gluconeogenesis occurring in the liver, plasma glucose concentrations are

increased for energy provision for cells that comprise tissues which do not store glycogen, for example, in the brain. Cortisol also acts at sites of inflammation where the higher temperature, known as pyrexia, can displace cortisol bound to corticosteroid-binding globulin (CBG). Furthermore, at such sites, the serine protease, neutrophil elastase, can cleave cortisol from CBG (Hammond *et al.*, 1990). Cortisol may then increase the transcription of anti-inflammatory cytokines, such as interleukin (IL)-1 receptor antagonist, which blocks the binding of pro-inflammatory IL-1 to IL-1 receptors (Levine *et al.*, 1996). Cortisol can also increase transcription of lipocortin-1, which inhibits phospholipase A_2 (Davidson *et al.*, 1987) and therefore limits the production of the lipid mediators, such as the prostaglandins and leukotrienes (derived from arachadonic acid) and lyso-platelet-activating factor. In addition cortisol can regulate inflammation by suppressing the synthesis of prostaglandins (Goppelt-Struebe, 1997), and cytokines such as IL-1 (Kern *et al.*, 1988), gamma-interferon (Arya *et al.*, 1984), IL-6 (Telleria *et al.*, 1998) and tumour necrosis factor-α (TNFα) (Beutler *et al.*, 1986). The majority of these cytokine genes, however, do not appear to have GREs in their promoter regions. Therefore, hormone-bound GRs appear to act by binding GREs in TFs such as nuclear factor-κB (NF-κB) or activator protein-1 (AP-1), that are involved in the recruitment of transcriptional co-activator proteins and induce chromatin modifications such as histone acetylation. So, GRs can decrease cytokine gene expression through cross-talk with, and transrepression of, NF-κB or AP-1 (Heck *et al.*, 1994; Liden *et al.*, 1997). Interestingly, GRs have also been shown to activate the synthesis of Iκ-B (Scheinman *et al.*, 1995), rendering NF-κB in an inactive form in the cytoplasm.

Dexamethasone, the synthetic glucocorticoid mentioned in section 1.2.4, has been shown to exert anti-apoptotic effects in granulosa cells *in vitro* by modulating the expression of members of the Bcl-2 family of proteins (Sasson and Amsterdam, 2002), which may be pro- or anti-apoptotic. In human granulosa cells, dexamethasone increased expression of Bcl-2 (anti-apoptotic), prevented the down-regulation of Bcl-xL (anti-apoptotic) and inhibited expression of Bak, Bax and Bcl-xS (pro-apoptotic) (Lotem and Sachs, 1995; Sakamoto *et al.*, 1995; Messmer *et al.*, 2000; Sasson *et al.*, 2001). Furthermore, dexamethasone could

block caspase-3 activation, which occurs in response to TNF-α and lipopolysaccharide (Messmer *et al.*, 2000).

1.3.3.1 Cortisol and female reproduction

Cortisol has been documented to have both positive and negative effects on ovarian function. As mentioned in section 1.2.4, a rise in glucocorticoid levels may suppress the GnRH pulse generator, GnRH and LH release and LH pulse frequency (Scholten and Liptrap, 1978; Dobson *et al.*, 1988; Dobson *et al.*, 2000) (Williams *et al.*, 1990; Ribadu *et al.*, 2000), which have been associated with COD. Dexamethasone appeared to activate the sympathetic nervous system in cystic ovaries and alter ovarian steroidogenesis (Jana *et al.*, 2005) and cortisol has been shown to inhibit LH-stimulated steroidogenesis in cultured human granulosa-lutein cells (Michael *et al.*, 1993b). Furthermore, in cultured rat granulosa cells, cortisol and dexamethasone were shown to prevent FSH-induced oestrogen production, by preventing the increase in aromatase activity induced by FSH (Hsueh and Erickson, 1978). In addition, cortisol has been shown to affect prostaglandin synthesis (reviewed by Goppelt-Struebe (1997)) and HAS-2 gene transcription (Stuhlmeier and Pollaschek, 2004), and prostaglandins and HA are both involved in cumulus expansion.

Cortisol concentrations were reported to be highest in FF from preovulatory human and bovine follicles (Harlow *et al.*, 1997; Yong *et al.*, 2000; Acosta *et al.*, 2005). One explanation for this may be that cortisol is displaced from CBGs by the higher concentrations of progesterone in these follicles (Andersen and Hornnes, 1994; Harlow *et al.*, 1997; Andersen, 2002). A second reason was proposed by Hillier and Tetsuka (1998), who hypothesised that cortisol may be required to limit the inflammation that occurs at ovulation (Espey, 1980). This will be discussed further in section 1.4.6. A final suggestion for the high levels of cortisol in preovulatory follicles relates to the effects of glucocorticoids on oocyte maturation, studies of which have shown varied results.

In a number of species of fish, cortisol has been shown to have positive effects on oocyte maturation (Kime *et al.*, 1992; Petrino *et al.*, 1993; Mugnier *et al.*, 1997) (Pinter and Thomas, 1999; Milla *et al.*, 2006; Mishra and Joy, 2006). In mouse oocytes, neither cortisol nor dexamethasone significantly altered the rate of GVBD, however dexamethasone appeared to decrease the percentage of oocytes progressing from GVBD to MII (Andersen, 2003). Both cortisol and dexamethasone inhibited GVBD in porcine COCs and denuded oocytes. The suppressive effects of cortisol and dexamethasone were abolished by the addition of the GR antagonist, RU-486 (Yang *et al.*, 1999), indicating that glucocorticoids inhibit the meiotic maturation of pig oocytes *in vitro*. Therefore, the negative effects of glucocorticoids on porcine oocyte maturation reported by Yang *et al.* (1999) may be species-specific.

The relationship between intrafollicular cortisol concentrations and the developmental potential of human oocytes has also been assessed. Andersen and Hornnes (1994) found no significant relationship between cortisol concentrations in follicles associated with pregnancy. Other papers found that conception through *in vitro* fertilization-embryo transfer (IVF-ET) positively correlated with high intrafollicular cortisol concentrations (Fateh *et al.*, 1989) (Keay *et al.*, 2002; Thurston *et al.*, 2003c). Some research groups also calculated the cortisol:cortisone ratios in FF (Michael *et al.*, 1999; Keay *et al.*, 2002; Lewicka *et al.*, 2003; Thurston *et al.*, 2003c), which reflect the inter-conversion of cortisol with its inactive metabolite, cortisone, catalysed by the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD). These groups detected significantly higher intrafollicular cortisol:cortisone ratios in women who conceived compared to women who did not. These results indicate that the metabolism of cortisol may increase the development potential of oocytes during IVF. The focus of this thesis is the metabolism of cortisol by 11βHSD in the porcine ovary and the functions of this enzyme will be discussed in section 1.4.

1.3.4 Metabolism of cortisol

Unbound cortisol can be metabolised by the action of enzymes, as mentioned above. The primary metabolism of cortisol occurs in the liver, prior to urinary excretion, where the steroid is required to become a hydrophilic compound, through the two-step reduction of the C=C double bonds in the first steroid ring. The first reduction reaction is catalysed by 5α- or 5β-reductase to produce allo-dihydrocortisol and dihydrocortisol, respectively. Then, 3α- or 3β-hydroxysteroid dehydrogenase (3α- or 3βHSD) reduces the ketone group of the dihydrocortisol compounds to form tetrahydrocortisol metabolites, which can become conjugated to glucuronic acid before elimination. Cortisone can also be modified by the above enzymes to ultimately form tetrahydrocortisone metabolites before excretion. Glucocorticoid metabolism action can also be regulated by the 11βHSD enzymes. The actions and functions of these enzymes will now be described.

1.4 11β-Hydroxysteroid Dehydrogenase (11βHSD)

1.4.1 Genes encoding the 11βHSD enzymes

The 11βHSD enzymes belong to the short-chain dehydrogenase/reductase (SDR) enzyme family. To date, two isoforms of 11βHSD (11βHSD1 and 11βHSD2) have been cloned, and the expression of a novel third isoform (11βHSD3) has been proposed. 11βHSDl is encoded by *HSD11B1*, which was first localised to chromosome 1 in humans by Tannin *et al.* (1991), and subsequently to human chormosome 1q32.2 (HSA1) (Goureau *et al.*, 1996). In the pig, *HSD11B1* was found on chromosome 9 (Otieno *et al.*, 2005), which has homology with human 1q. The rat and human genes encoding 11βHSD1 have six exons spanning 30 kilobases (kb) in length (Krozowski *et al.*, 1990; Obeid *et al.*, 1993; White *et al.*, 1994). The human 11βHSD1 enzyme protein consists of 292 amino acids with a molecular weight (MW) of 34 kilodaltons (kDa). A complimentary deoxyribonucleic acid (cDNA) encoding 11βHSD1 was first cloned from rat liver (Agarwal *et al.*, 1989) before human (Agarwal *et al.*, 1989), sheep (Yang *et al.*, 1992), squirrel monkey (Moore *et al.*, 1993) and mouse (Rajan *et al.*, 1995) cDNAs were cloned. The encoded 11βHSD1 amino acid sequences from these

species exhibit 71-91% homology with each other (Tannin *et al.*, 1991; Yang *et al.*, 1992; Moore *et al.*, 1993; Rajan *et al.*, 1995).

The gene encoding 11βHSD2, named *HSD11B2* (Krozowski *et al.*, 1995b), was initially cloned from the sheep (Agarwal *et al.*, 1994) and human (Albiston *et al.*, 1994). The human gene was localised to chromosome 16q22 (Agarwal *et al.*, 1995; Krozowski *et al.*, 1995b). At 6.2 kb in length, with five exons, *HSD11B2* is shorter than *HSD11B1*, however human 11βHSD2 consists of 405 amino acids with a MW of 44 kDa. Porcine 11βHSD2 contains 400 amino acids (Lange *et al.*, 2003). The human, sheep and rabbit amino acid sequences for 11βHSD2 share 75-87% identity (Krozowski *et al.*, 1995a). There is only 14% homology between human 11βHSD1 and 11βHSD2 (Albiston *et al.*, 1994).

Recent literature has been published describing evidence for a new isoform of 11βHSD, which was provisionally named 11βHSD3 (Gomez-Sanchez *et al.*, 1997). This novel isoform has not yet been cloned nor has its genetic location been published. However, the primary sequences for 11βHSD3 from 6 individual species (Japanese killifish, three-spined stickleback, zebrafish, chicken, cow and long-tailed macaque) have been cloned and these sequences have been deposited in the NCBI protein database by Huang C. The existence of this third enzyme was suggested in human granulosa-lutein cells (Michael *et al.*, 1997), and has only been identified through 11βHSD activity studies carried out in the sheep and rat (Ge *et al.*, 1997; Gomez-Sanchez *et al.*, 1997; Ge and Hardy, 2000).

1.4.2 Biochemistry and molecular biology of 11βHSD actions

1.4.2.1 Membrane orientation and conserved sequences in the 11βHSD enzymes

Ozols (1995) first discovered specific disulphide bonds and a glycosylation pattern in the structure of 11βHSD1 to suggest that it was expressed in the smooth endoplasmic reticulum (SER). Later, both 11βHSD1 and 11βHSD2 were shown to have a transmembrane domain at the N-terminus, anchored to the SER membrane,

and a catalytic site in the C-terminus (Odermatt *et al.*, 1999). 11βHSD1 and 11βHSD2 contain one and three transmembrane helices, respectively, therefore the active site of 11βHSD1 faces the SER lumen (Ozols, 1995; Mziaut *et al.*, 1999; Odermatt *et al.*, 1999), whereas that of 11βHSD2 faces the cytoplasm (Odermatt *et al.*, 1999). The importance of the location of 11βHSD1 in the SER will be discussed further in section 1.4.2.2.

The active site of SDRs contains a conserved motif of Tyr-X-X-X-Lys (Penning, 1997), and the 11βHSD enzymes possess a catalytic triad of serine, tyrosine and lysine residues to perform oxidoreductase enzyme reactions (Liu *et al.*, 1997; Penning, 1997). The site-directed mutation of the Tyr-179 and Lys-183 in this motif of human 11βHSD1 abolished enzyme activity (Obeid and White, 1992). The hydroxyl group of the serine binds to the target side-chain of the steroid to stabilise the substrate. The lysine binds to the cofactor and lowers the pKa of the phenolic hydroxyl group of the tyrosine, which can then function as a weak acid and donate protons (Filling *et al.*, 2002). All SDRs also have a common Gly-X-X-X-Gly-X-Gly motif in the N-terminus, giving rise to a Rossmann fold, to which pyridine nucelotide cofactors can bind. This protein fold contains a seven-stranded parallel β-sheet with three α-helices on either side (see review by Krozowski (1994)). The different cofactors needed to drive the activities of the enzymes will be described next.

1.4.2.2 Cofactor preference and catalytic activity of the 11βHSD enzymes

Both cloned 11βHSD enzymes (11βHSD1 and 11βHSD2) can mediate the modification of the hydroxyl group at carbon 11 of cortisol to a ketone group in cortisone, through the 11β-dehydrogenase (11β-DH) activities of the enzymes. 11βHSD1 can additionally catalyse the reverse direction of reaction, the regeneration of cortisol from cortisone, via its 11-ketosteroid reductase (11-KSR) activity (Lakshmi and Monder, 1985; Lakshmi and Monder, 1988).

The 11βHSD1 enzyme utilises the oxidised and reduced isoforms of nicotinamide adenine dinucleotide phosphate ($NADP^+$ and NADPH) to support the 11β-DH and 11-KSR activities, respectively. 11βHSD1 may also use the isoforms of nicotinamide adenine dinucleotide (NAD(H)), which, being a smaller pyridine nucleotide, could occupy the Rossman fold. *In vivo*, 11βHSD1 has a higher affinity for cortisone (K_m = 300nM) than cortisol (K_m = 27μM) and the 11-KSR activity of 11βHSD1 in the SER appears to be driven in most cells by hexose-6-phosphate dehydrogenase (H6PDH) (Draper *et al.*, 2003; Atanasov *et al.*, 2004; Bujalska *et al.*, 2005; Czegle *et al.*, 2006; Odermatt *et al.*, 2006; White *et al.*, 2007) (Figure 1.5). H6PDH catalyses the first two steps of the pentose phosphate pathway, yielding NADPH (Ozols, 1993). One of the H6PDH-catalysed reactions is the conversion of glucose-6-phosphate (G-6-P) to 6-phosphogluconate. The co-operation of H6PDH and 11βHSD1 was first indicated when the two enzymes co-localised in human embryonic kidney- (HEK)-293 cells (Atanasov *et al.*, 2004). In Chinese hamster ovary (CHO) cells, increasing H6PDH mRNA levels increased the 11-KSR activity of 11βHSD1, while decreasing 11β-DH activity (Bujalska *et al.*, 2005). Recently, H6PDH knock-out mice were shown to be unable to regenerate the primary murine glucocorticoid, corticosterone (Lavery *et al.*, 2006).

In steroidogenic cells however, such as those of the ovary and testis, 11βHSD1 appears to act as an 11β-DH (Gao *et al.*, 1997; Michael *et al.*, 1997; Ge and Hardy, 2000; Yong *et al.*, 2000; Tetsuka *et al.*, 2003; Thurston *et al.*, 2007). In these cells, NADPH may be primarily utilised by cytochrome P450 (CYP) enzymes, which are also located in the SER of steroidogenic cells (Balasubramaniam *et al.*, 1981; Ishimura and Fujita, 1997), increasing the availability of $NADP^+$ to drive cortisol inactivation by 11βHSD1 (Michael *et al.*, 2003; Ge *et al.*, 2005) (Figure 1.5). In bovine granulosa cells, 11βHSD1 expression negatively was found to be correlated with intrafollicular cortisol concentrations, suggesting that 11βHSD1 acted as an 11β-DH in the bovine follicle (Tetsuka *et al.*, 2003). Subsequently, Thurston *et al.* (2007) showed that predominant dehydrogenase activities could be observed in bovine follicular granulosa cells isolated and maintained in primary culture.

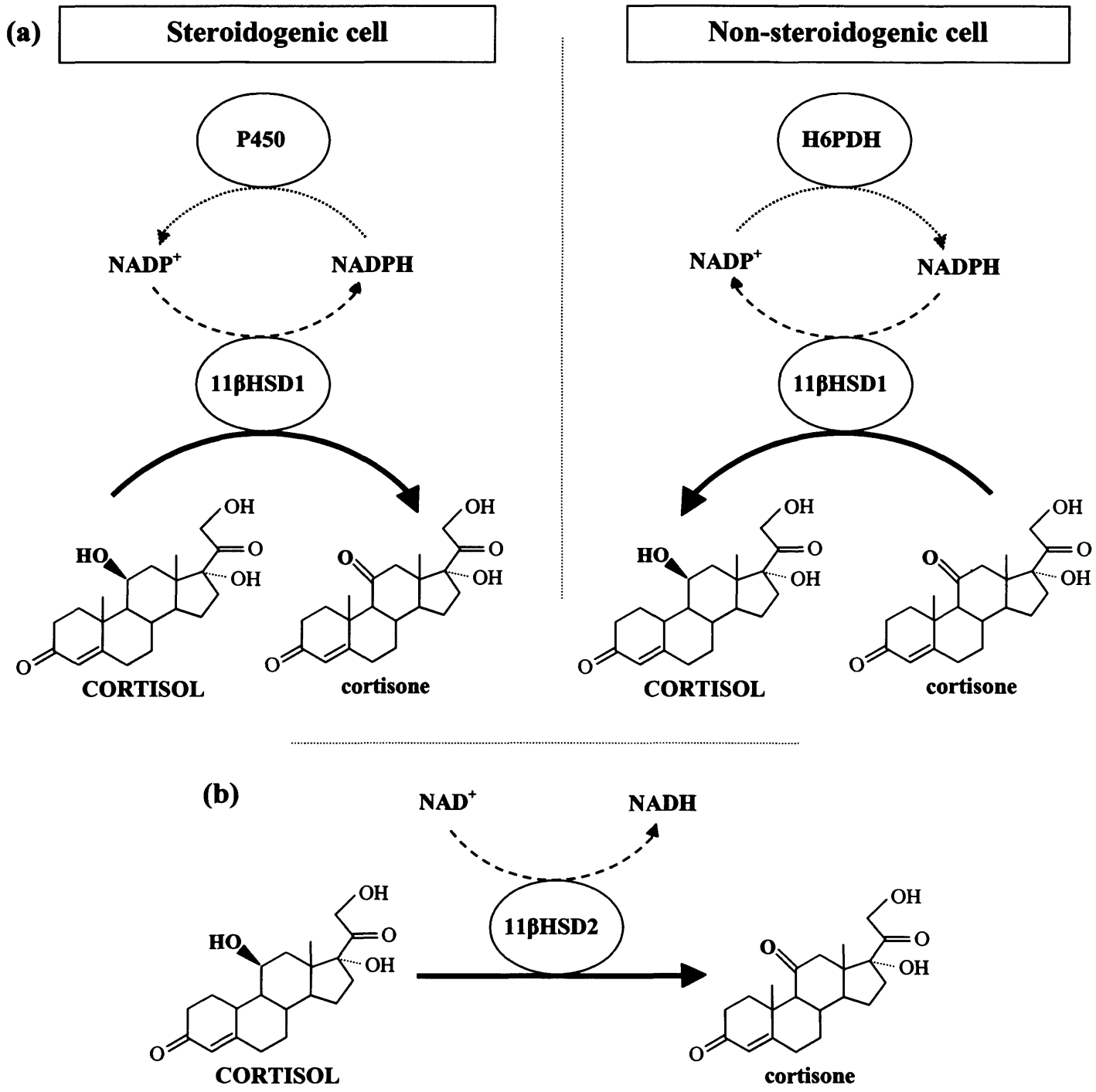


Figure 1.5. Cofactor selectivity and reaction mechanisms for (a) 11βHSD1 and (b) 11βHSD2 enzymes. In the top diagrams, the proposed mechanisms for 11βHSD1 in steroidogenic and non-steroidogenic cells are indicated. In the upper reaction schemes, arrows with dotted black lines represent predominant $NADP^+$ production by the cytochrome P450 enzymes to drive the 11β-DH activity of 11βHSD1 in steroidogenic cells and conversely, the predominant production of NADPH by H6PDH to drive the 11-KSR activity of 11βHSD1 in non-steroidogenic cells. The 11βHSD2 enzyme however, only utilises NAD^+ and thus demonstrates exclusive 11β-DH activity.

Unlike 11βHSD1, 11βHSD2 has a high affinity for cortisol (K_m = 14-60nmol l^{-1}) (Brown *et al.*, 1993; Albiston *et al.*, 1994) and only utilises the oxidised form of nicotinamide adenine dinucleotide (NAD^+). Thus, 11βHSD2 exclusively inactivates cortisol (Mercer and Krozowski, 1992; Brown *et al.*, 1993; Agarwal *et al.*, 1994; Albiston *et al.*, 1994). *In vitro* studies of rabbit and rat tissues have shown that, in the presence of excess concentrations of 11-dehydro-corticosterone (the 11-ketosteroid metabolite of corticosterone in these species), the dehydrogenase activity of 11βHSD2 was subject to end-product inhibition (Rusvai and Naray-Fejes-Toth, 1993). Only the $NADP^+$-dependent activities of 11βHSD3 have been documented to date. In the sheep kidney, 11βHSD3 had a greater affinity for cortisol (K_m = 35.2±2nM) than 11βHSD1 (Gomez-Sanchez *et al.*, 1997) to drive cortisol inactivation. A similar substrate affinity was identified in rat Leydig cells, where this third enzyme isoform could catalyse $NADP^+$-dependent inactivation of corticosterone (K_m = 42nM) (Ge *et al.*, 1997; Ge and Hardy, 2000).

1.4.3 Sites of expression for the 11βHSD isoforms

Initially, 11βHSD1 was isolated from the liver by Lakshmi and Monder (1988), before Monder and Lakshmi (1990) detected 11βHSD1 expression in glucocorticoid target tissues in the rat, such as the kidney, lung, brain, and heart. 11βHSD1 was later shown to be ubiquitously expressed in human tissues (Ricketts *et al.*, 1998). 11βHSD2 did not share this ubiquitous pattern of expression however, and was found in mineralocorticoid target tissues, specifically, the distal nephron of the kidney, colon, pancreas, salivary glands, as well as in the prostate and placenta (Naray-Fejes-Toth *et al.*, 1991; Mercer and Krozowski, 1992; Brown *et al.*, 1993; Albiston *et al.*, 1994; Ricketts *et al.*, 1998).

The expression of the 11βHSD isoforms in the cells of the ovary is of particular relevance to this project, and a summary of expression is shown in Table 1.1. 11βHSD1 was first identified in human ovarian tissue (Tannin *et al.*, 1991), and

Table 1.1. Overview of the expression of 11βHSD enzymes reported in the ovary

Ovarian cell	Species	Enzyme isoform	Reference
Ovarian surface epithelium	Human	11βHSD1	(Yong et al., 2002) (Rae et al., 2004) (Gubbay et al., 2004)
Theca cells	Human	11βHSD2	(Ricketts et al., 1998)
Follicular granulosa cells	Human	11βHSD2	(Tetsuka et al., 1997) (Michael et al., 1997) (Ricketts et al., 1998)
	Rat	11βHSD2	(Tetsuka et al., 1999b) (Tetsuka et al., 1999a)
	Cow	11βHSD1 + 11βHSD2*	(Tetsuka et al., 2003) (Thurston et al., 2007)
Granulosa-lutein cells	Human	11βHSD1	(Ricketts et al., 1998)
	Rat	11βHSD1	(Tetsuka et al., 1999b) (Tetsuka et al., 1999a)
	Cow	11βHSD1* + 11βHSD2	(Tetsuka et al., 2003) (Thurston et al., 2007)
Cumulus cells	Human	11βHSD1	(Smith et al., 2000)
Oocyte	Human	11βHSD1	(Ricketts et al., 1998) (Smith et al., 2000)
	Rat	11βHSD1	(Benediktsson et al., 1992)

* The predominant enzyme isoform in cells where co-expression of both 11βHSD enzymes occurs.

later in the rat CL and oocyte (Benediktsson *et al.*, 1992), human oocytes (Ricketts *et al.*, 1998; Smith *et al.*, 2000) and human ovarian surface epithelial cells (Yong *et al.*, 2002). In human ovarian follicles, only 11βHSD2 expression was detected in the theca cells (Ricketts *et al.*, 1998), but the expression of 11βHSD enzymes in the granulosa cells has been shown to change with the stage of the ovarian cycle (Michael *et al.*, 1997) (Tetsuka *et al.*, 1997; Ricketts *et al.*, 1998; Tetsuka *et al.*, 1999a; Tetsuka *et al.*, 1999b). 11βHSD2 is exclusively expressed in granulosa cells prior to ovulation, whereas only 11βHSD1 is expressed in luteinising granulosa cells. This finding, along with the expression reported in oocytes, suggests that 11βHSD plays a role in folliculogenesis and ovulation. This will be discussed further in section 1.4.6.

1.4.4 Physiological functions of the 11βHSD enzymes

As mentioned in section 1.4.2.2, 11βHSD1 appears to predominantly reactivate cortisol in most tissues, due to the higher affinity of the enzyme for cortisone and possibly due to the provision of NADPH as a result of the intralumenal activity of H6PDH. Predominant cortisol reactivation could maximise the activation of the GRs (reviewed by Seckl and Walker (2001)) and in turn, increase gluconeogenesis, particularly during times of stress.

Defects in 11βHSD1 can cause clinical syndromes such as apparent cortisone reductase deficiency (ACRD), where the regeneration of cortisol from cortisone is impaired (Phillipou and Higgins, 1985; Phillipov *et al.*, 1996). Females with ACRD may suffer from anovulatory infertility, polycystic ovaries, hirsutism and hyperandrogenism (Phillipou and Higgins, 1985; Jamieson *et al.*, 1999). These symptoms are similar to those seen in women with polycystic ovary syndrome (PCOS), which will be mentioned below.

Sufferers of ACRD were found to have no mutations in the promoter, exons or regulatory elements of the *HSD11B1* gene (Nikkila *et al.*, 1993; Jamieson *et al.*, 1999). Mutations were later discovered in the introns of *HSD11B1* in three ACRD

patients, which may decrease *HSD11B1* gene transcription (Draper *et al.*, 2003). These affected individuals also had mutations in exon 5 of the gene encoding H6PDH, which may have additionally impaired H6PDH enzyme activity and therefore limited the intralumenal supply of NADPH to drive the the 11-KSR activity of 11βHSD1.

PCOS sufferers show symptoms of hirsutism and amenorrhoea (reviewed by Franks (1989) and Franks *et al.* (2006)). These symptoms have been linked to hyperandrogenism (Conway *et al.*, 1989) which could be associated with altered cortisol metabolism and, potentially, impaired 11βHSD1 function (Stewart *et al.*, 1993; Walker, 2001; Tsilchorozidou *et al.*, 2003; Gambineri *et al.*, 2006). Normally, cortisol exerts negative feedback on CRH and ACTH production, however, if there is a defect in cortisol regeneration, CRH and ACTH levels may continue to rise. Recent studies involving 11βHSD1 knockout mice have demonstrated that the reductase activity of 11βHSD1 in the hippocampus and/or anterior pituitary appears to be required for glucocorticoids to exert full negative feedback in the HPA axis (Kotelevtsev *et al.*, 1997; Harris *et al.*, 2001). A rise in CRH and ACTH may induce an increase in the production of adrenal androgens by the ZR and/or androgens by the theca cells of the ovary, possibly leading to hyperandrogenism. 11βHSD1 knockout mice could not regenerate corticosterone but had increased plasma corticosterone levels. This was attributed to a decreased negative feedback on CRH and ACTH synthesis in the HPA axis due to a lack of glucocorticoid regeneration by 11βHSD1. The increased ACTH levels, however, could then stimulate glucocorticoid production in the ZF. Interestingly, female mice were fertile, though it has been established that women with ACRD, who lack the 11-KSR activity of 11βHSD1, may be anovulatory and infertile.

11βHSD2 expression has been documented at sites of MR localisation, for example, the distal convoluted tubules and collecting ducts of the nephrons in the kidney (Edwards *et al.*, 1988; Funder *et al.*, 1988; Cole, 1995; Krozowski *et al.*, 1995c; Bostanjoglo *et al.*, 1998). This 11βHSD isoform is also expressed in the colon and salivary glands, which are also mineralocorticoid-target tissues, as

described in secton 1.4.3. The human MR was shown to have the same affinity for cortisol and aldosterone (Arriza *et al.*, 1987) however, physiological levels of cortisol (nM) are 1000-fold greater than aldosterone (pM). 11βHSD2 must therefore inactivate cortisol to protect MRs from excessive stimulation (Edwards *et al.*, 1988; Funder *et al.*, 1988). 11βHSD2 decreases cortisol concentrations by around 90%, so cortisol levels are 10-fold higher than aldosterone (Funder, 2005). Funder's recent calculations indicate that even in the presence of 11βHSD2, cortisol and aldosterone stimulate the MR in an approximately 1:1 ratio, unless 11βHSD2 activity is exceeded or compromised.

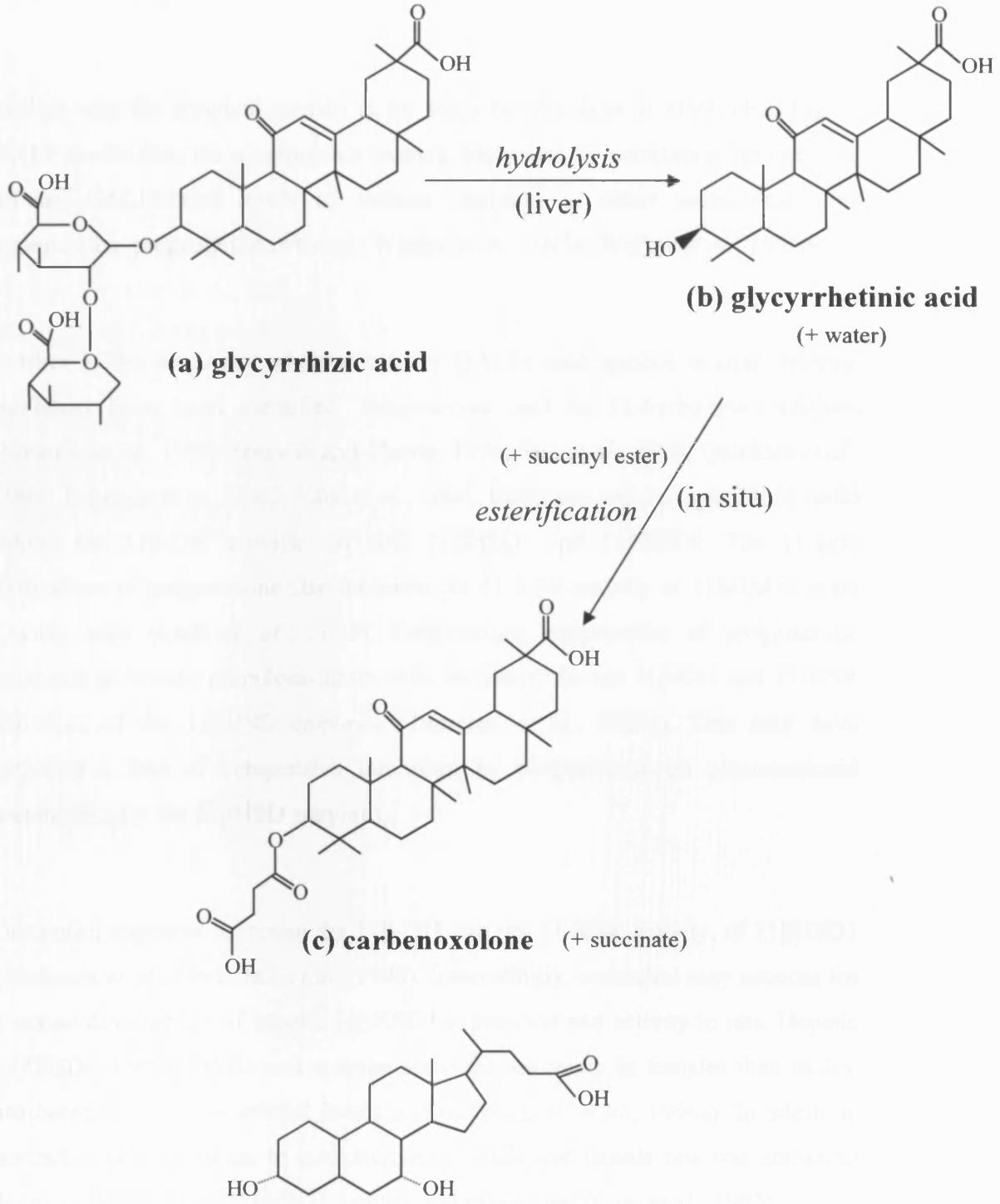
Apparent mineralocorticoid excess (AME) is one syndrome caused by a defect in 11βHSD2 activity. As the MRs regulate the retention of reabsorption of Na^+ ions and water, and control the loss of K^+ ions AME symptoms include hypertension and hypokalemia. The first reports of AME were in children who had hypertension and low levels of both plasma aldosterone and urinary cortisone metabolites (Ulick *et al.*, 1979), the latter suggesting a lack of cortisol inactivation. Therefore, AME is caused by overactivation of MRs due to the excess actions of glucocorticoids, not mineralocorticoids (Edwards *et al.*, 1988; Funder *et al.*, 1988; Stewart *et al.*, 1988). Transgenic 11βHSD2 null mice exhibited hypertension, hypotonic polyuria and hypokalemia (Holmes *et al.*, 2001), corresponding with the observed symptoms of AME.

There are two sub-types of AME: type 1 AME is caused by mutations in the *HSD11B2* gene, inherited as a recessive mutation (Mune *et al.*, 1995; Ferrari *et al.*, 1996). This is very rare with less than a hundred individuals affected. Type 2 AME occurs through an inhibition of the 11βHSD2 enzyme, potentially by dietary compounds. The inhibitors of the 11βHSD enzymes are relevant to this thesis and will be discussed next.

1.4.5 Regulation of 11βHSD activity by enzyme inhibitors

The first reported exogenous inhibitors of the 11βHSD enzymes were glycyrrhizic acid (GZ) and glycyrrhetinic acid (GA), (Stewart *et al.*, 1987; Monder *et al.*, 1989), the components of liquorice root. GZ has a disaccharide group at carbon 3 which can be hydrolysed in the liver and kidney to form GA, with a hydroxyl group at carbon 3 (Figure 1.6). These compounds can inhibit 11βHSD1 and 11βHSD2 activities in rat tissues (Monder *et al.*, 1989; Irie *et al.*, 1992; Marandici and Monder, 1993), and human kidney (Stewart *et al.*, 1987; Kageyama *et al.*, 1992). GA also decreases 11βHSD2 expression in mineralocorticoid (kidney and colon) and glucocorticoid (liver and pituitary) target tissues of the rat both *in vivo* and *in vitro* (Whorwood *et al.*, 1993). Carbenoxolone (CBX) is a synthetic succinyl ester of GA (Figure 1.6), and a potent inhibitor of both 11βHSD1 and 11βHSD2 activities. CBX decreased the renal 11β-DH activity of 11βHSD1 of the rat kidney *in vivo* (Funder *et al.*, 1988). CBX could also inhibit the bi-directional 11β-DH and 11-KSR activities of 11βHSD1 (Stewart *et al.*, 1990).

Physiological compounds, such as the bile acids and their mono-, di- and trihydroxylated bile acid derivatives, have also been reported to have inhibitory effects on 11βHSD activities (Perschel *et al.*, 1991; Latif *et al.*, 1994). One such bile acid, chenodeoxycholic acid (CDCA; Figure 1.6), is structurally similar to GA and was shown to inhibit both the 11β-DH and 11-KSR activities of 11βHSD1 and the oxidative activity of 11βHSD2 (Diederich *et al.*, 2000; Morris *et al.*, 2004). Endogenous inhibitors of 11βHSD1 have also been found in the urine of normotensive men, and both nonpregnant and pregnant women. These inhibitors have not yet been identified nor their origin ascertained, but as these compounds acted in a manner similar to GA, they were named glycyrrhetinic acid-like factors (GALFs). Levels of GALFs were higher in pregnant than nonpregnant women, and both groups of women had a higher GALF content than normotensive men (Morris *et al.*, 1992). A group of patients with hypertension were later found to have elevated levels of urinary GALFs (Walker *et al.*, 1995a), providing a potential link of these enzyme inhibitors to type 2 AME. Interestingly, pregnant women can experience gestational hypertension, preeclampsia or eclampsia, which are all accompanied by an increase in sodium levels. This may



(a) glycyrrhizic acid

(b) glycyrrhetinic acid

(c) carbenoxolone

(d) chenodeoxycholic acid

Figure 1.6. Chemical structures for the 11βHSD enzyme inhibitors: (a) glycyrrhizic acid (GZ), (b) glycyrrhetinic acid (GA), (c) carbenoxolone (CBX) and (d) chenodeoxycholic acid (CDCA). The arrows indicate the relationships between CZ, GA and CBX: GZ is hydrolysed to GA in the liver, and CBX is a succinyl ester derivative of GA produced in the laboratory. Reaction types are shown in italic font.

explain why the pregnant women in the study by Morris *et al.* (1992) had higher GALF levels than the nonpregnant women. However, no correlation between the urinary GALFs and levels of sodium retention in either normotensive or hypertensive pregnancy was found (Walker *et al.*, 1995a; Walker *et al.*, 1995b).

Further to the discovery of these urinary GALFs, endogenous ovarian enzyme inhibitors have been identified. Progesterone and its 11-hydroxy-metabolites (Souness *et al.*, 1995; Souness and Morris, 1996; Sun *et al.*, 1998; Quinkler *et al.*, 1999; Thurston *et al.*, 2002; Latif *et al.*, 2005; Robinzon and Prough, 2005) could inhibit the 11β-DH activities of both 11βHSD1 and 11βHSD2. The 11-keto derivatives of progesterone also inhibited the 11-KSR activity of 11βHSD1 in rat Leydig cells (Latif *et al.*, 2005). Furthermore, suppression of progesterone synthesis in human granulosa-lutein cells increased the net 11β-DH and 11-KSR activities of the 11βHSD enzymes (Thurston *et al.*, 2003a). This may have reflected a loss of competitive inhibition by progesterone on glucocorticoid metabolism by the 11βHSD enzymes.

Oestradiol appeared to inhibit the 11β-DH, but not 11-KSR activity, of 11βHSD1 (Jamieson *et al.*, 1995; Sun *et al.*, 1998). Interestingly, oestradiol may account for a sexual dimorphism of hepatic 11βHSD1 expression and activity in rats. Hepatic 11βHSD1 mRNA levels and enzyme activities are lower in females than males, attributed to higher oestradiol levels in females (Low *et al.*, 1994a). In addition, oestradiol administration to gonadectomised male and female rats was shown to decrease both hepatic 11βHSD1 activity and expression (Low *et al.*, 1993).

As well as oestradiol production, gender-specific GH release could alter the expression of rat hepatic proteins between males and females (Wiwi and Waxman, 2004). Hepatic 11βHSD1 may be one example, such that the effects of oestradiol on 11βHSD1 expression may be GH-mediated. This concept arose from the observation that oestradiol administration to GH-deficient (dwarf) rats did not alter hepatic 11βHSD1 expression or activity (Low *et al.*, 1994a) though administration of GH itself decreased basal hepatic 11βHSD1 activity and

expression. In a similar study carried out in GH-deficient patients, GH-replacement therapy suppressed hepatic 11βHSD1 activity (Walker *et al.*, 1998). Subsequently, Moore *et al.* (1999) found that IGF-1 could decrease 11βHSD1 activity in a concentration-dependent manner. As GH was shown to stimulate the *in vivo* production of IGF-1 in the rat liver, kidney and ovary (Davoren and Hsueh, 1986; Maiter *et al.*, 1992), GH may mediate effects on 11βHSD1 through IGF-1. GH was recently found to decrease *in vitro* 11βHSD1 mRNA production while increasing that of IGF-1 (Paulsen *et al.*, 2006). Therefore, in summary, the inhibitory effects of oestradiol on 11βHSD1 expression and activity may be mediated via the action of IGF-1 which, in turn, may be regulated by GH.

One further ovarian inhibitor of 11βHSD1 activity was previously found by our laboratory in FF from the large antral follicles of pigs, cows and women and in fluid from the ovarian cysts of pigs and cows (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). These intrafollicular compounds are the focus of this thesis and will be discussed in section 1.4.7.

1.4.6 The importance of 11βHSD in the ovary

1.4.6.1 11βHSD enzyme expression in ovarian cells

As mentioned in section 1.4.3, 11βHSD1 and 11βHSD2 are expressed in the granulosa cells at different phases of the ovarian cycle (Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Ricketts *et al.*, 1998; Tetsuka *et al.*, 1999a; Tetsuka *et al.*, 1999b; Tetsuka *et al.*, 2003; Thurston *et al.*, 2003a). In the human (Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Ricketts *et al.*, 1998) and rat (Tetsuka *et al.*, 1999a; Tetsuka *et al.*, 1999b), there is exclusive 11βHSD2 expression in granulosa cells during the follicular phase of the ovarian cycle, whereas luteinising granulosa cells only express 11βHSD1. In the cow, however, both 11βHSD isoforms are co-expressed during both phases of the cycle. There is predominant 11βHSD2 expression over 11βHSD1 in bovine follicular granulosa cells and 11βHSD1 expression in the active CL, with 11βHSD2 expression dominating in the regressing corpus albicans (Tetsuka *et al.*, 2003; Thurston *et al.*, 2007).

Given that ovulation is an inflammatory event (Espey, 1980), Hillier and Tetsuka (1998) suggested that the change of 11βHSD isoform expression in the granulosa cells with the phase of the ovarian cycle could serve to limit the inflammation arising at ovulation. The expression of 11βHSD1 in granulosa cells at the time of ovulation could increase local levels of cortisol which, as discussed in section 1.3.3, is anti-inflammatory (Hillier and Tetsuka, 1998; Andersen, 2002). Cortisol has indeed been shown to inhibit the synthesis of both prostaglandins and of pro-inflammatory cytokines within the ovary (Goppelt-Struebe, 1997; Telleria *et al.*, 1998). It has also been demonstrated that pro-inflammatory interleukins produced prior to ovulation, such as IL-1β, as well as LH itself, can upregulate 11βHSD1 expression in the granulosa cells (Evagelatou *et al.*, 1997; Tetsuka *et al.*, 1999a). As discussed in section 1.2.2, LH also upregulates the expression of PGHS-2 in the cumulus cells to increase prostaglandin production at the time of cumulus expansion and ovulation (Morris and Richards, 1993). Recently, Jonas *et al.* (2006) showed that suppression of prostaglandin production by pharmacological inhibitors, in turn, decreased both the dehydrogenase and reductase activities of 11βHSD1 in human granulosa-lutein cells. This raises the possibility that the increase in prostaglandin production at ovulation also modulates 11βHSD1 activities in the granulosa cells.

The alteration of 11βHSD isoform expression shown in rat, cow and human granulosa cells across the ovarian cycle may also result from the change in predominant hormone production during each phase of the cycle. During the follicular phase, there is almost exclusive oestrogen output by the granulosa cells. As described above, oestradiol appeared to suppress hepatic 11βHSD1 mRNA levels and enzyme activities (Low *et al.*, 1993; Low *et al.*, 1994a). If the negative effects of oestradiol on 11βHSD1 expression and activity were observed in ovarian cells during the follicular phase, this might explain the relatively higher levels of 11βHSD2 expression observed in follicular granulosa cells.

After the follicular phase, however, the LH surge may upregulate 11βHSD1 expression in the granulosa cells (Tetsuka *et al.*, 1999a). Progesterone has also been shown to induce 11βHSD1 expression in the rat uterus during pregnancy (Burton *et al.*, 1996). In the rat CL, 11βHSD1 was expressed at high levels until luteolysis, when the expression of 11βHSD1 decreased and that of 11βHSD2 rose, coinciding with the loss of progesterone synthesis (Waddell *et al.*, 1996). Despite this, in cultured human granulosa-lutein cells undergoing luteinisation, 11βHSD1 expression was not affected by progesterone suppression but enzyme expression did increase as granulosa cells luteinised (Thurston *et al.*, 2003a). Therefore, the upregulation of 11βHSD1 expression in luteinising cells may not solely rely on local effects of progesterone.

Further to this finding, the fact that both 11βHSD1 and 11βHSD2 expression can be observed in bovine granulosa cells at all times of the ovarian cycle also suggests that enzyme expression does not depend exclusively on follicular or luteal steroid production. In bovine follicular granulosa cells, despite high levels of oestradiol production, 11βHSD1 expression can be observed. Likewise in bovine granulosa-lutein cells, high progesterone output coincided with predominant 11βHSD2 expression in the corpus albicans (Tetsuka *et al.*, 2003).

1.4.6.2 11βHSD activities in ovarian cells

The conversion of cortisol to cortisone, and vice versa, was first demonstrated in ovarian homogenates by Murphy (1981). In the foetal gonad, percentage conversion of cortisol to cortisone (35%) outweighed cortisone-cortisol conversion (6%). This was suggested to limit foetal exposure to maternal glucocorticoids, which induce premature differentiation (Kitanaka *et al.*, 1996). In the adult ovary, conversion of cortisol to cortisone was 28%, compared to the 23% conversion of cortisone to cortisol.

The metabolism of cortisol in human granulosa-lutein cells was first reported by Owen *et al.* (1992). After incubation of granulosa cells with radiolabelled cortisol, radiolabelled 11β-hydroxyandrostenedione was detected in the cells, thus, 11β-

hydroxyandrostenedione appeared to have formed from cortisol. This reaction, however, is likely to have been catalysed by the 17,20-lyase activity of CYP17, due to the presence of theca cells in the culture. Radiolabelled cortisone was also detected in the cells, though this was not discussed in the paper as being catalysed by one of the 11βHSD enzymes.

Michael *et al.* (1993b) also measured cortisol oxidation by 11βHSD in granulosa-lutein cells of women undergoing IVF-ET, where cells exhibiting high 11βHSD activities were referred to as "11βHSD-positive" cells and cells displaying low 11βHSD activities were described as "11βHSD-negative" cells. This study showed that cortisol could inhibit LH-stimulated steroidogenesis by the human granulosa-lutein cells, irrespective of levels of basal intracellular 11βHSD activity. However, higher concentrations of cortisol were required to have suppressive effects on steroidogenesis in "11βHSD-positive" than in "11βHSD-negative" cells. The IC_{50} value for cortisol in "11βHSD-positive" cells was 1250±377nM, whereas the IC_{50} for cortisol in "11βHSD-negative" cells was 158±41nM.

In a subsequent study, it was shown 63% of women undergoing IVF-ET who had "11βHSD-negative" granulosa lutein cells (undetectably low 11βHSD activities) became pregnant, though none of the women who possessed "11βHSD-positive" cells (high 11βHSD activities) conceived (Michael *et al.*, 1993a). As hypothesised in section 1.4.2.2, if steroidogenic cells displayed predominant 11β-DH activities, the low enzyme activities in the "11βHSD-negative" cells may manifest as high intrafollicular cortisol:cortisone ratios, which were also significantly correlated with the probability of conception occurring through IVF-ET (Michael *et al.*, 1999; Keay *et al.*, 2002; Lewicka *et al.*, 2003; Thurston *et al.*, 2003c).

In follicular granulosa cells from small human antral follicles, there was 42% conversion of cortisol to cortisone, compared to 1% conversion of cortisone to cortisol (Yong *et al.*, 2000) indicating the exclusive dehydrogenase activities of 11βHSD in human follicular granulosa cells. Granulosa cells from preovulatory

follicles possessed similar 11β-DH activities to small follicles (32% conversion of cortisol to cortisone) but higher 11-KSR activities (36% conversion of cortisone to cortisol) and higher cortisol:cortisone ratios than small follicles. The increasing 11-KSR activities in large follicles may demonstrate the upregulation of 11βHSD1 expression as the follicle approaches ovulation and the luteal phase.

1.4.7 Regulation of 11βHSD activity in the ovary

Michael *et al.* (1996) first suggested that human granulosa-lutein cells may produce a paracrine factor that could suppress net cortisol oxidation by 11βHSD in other granulosa-lutein cells in culture. The activities of 11βHSD in granulosa-lutein cells from single ovarian follicles were measured and compared with the enzyme activities in cells pooled from several different combinations of these follicles. The activities of 11βHSD in pooled granulosa-lutein cells were consistently lower than mean enzyme activities calculated for the respective individual follicles. This raised the possibility that in a pooled-cell culture "11βHSD-negative" cells (those that displayed very low 11βHSD activities when cultured alone) may have been suppressing enzyme activities in "11βHSD-positive" cells (those that exhibited high 11βHSD activities when cultured separately).

Intrafollicular compounds that can inhibit 11βHSD1 activities have recently been found in the fluid from large antral follicles of pigs, cows and women and in the fluid from spontaneous ovarian cysts of pigs and cows ((Thurston *et al.*, 2002; Thurston *et al.*, 2003b), section 1.4.5). Samples of FF and cyst fluid were initially tested for effects on the activities of both 11βHSD1 and 11βHSD2 in male rat kidney homogenates, since male rat kidney expresses both cloned enzymes (Low *et al.*, 1993). Compounds in FF and cyst fluid appeared to selectively alter the activities of 11βHSD1, with no effects on 11βHSD2 activities, since NADP(H)-, but not NAD^+-dependent, enzyme activities were affected. While urinary GALFs share similar biophysical properties with the ovarian inhibitors of 11βHSD1 (Thurston *et al.*, 2002), it is not known whether these two sub-groups of enzyme inhibitors are related.

Further examination of the ovarian 11βHSD1 modulators in FF from preovulatory porcine, bovine and human follicles indicated that two classes of regulatory compounds existed. These ovarian fluids contained both hydrophilic compounds, which acted as enzyme stimulators and increased NADP(H)-dependent 11βHSD activity by up to three fold in 1 hour, and hydrophobic compounds could inhibit enzyme activity by up to 84% (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). The levels of the intrafollicular hydrophobic inhibitors were linked to the probability of conception occurring through IVF-ET (Thurston *et al.*, 2003c). As mentioned above, the high intrafollicular ratios of cortisol:cortisone that correlated with the probability of conception could be attributed to low 11β-DH activities in the follicle. The low enzyme activities could, in turn, be correlated with high levels of the hydrophobic inhibitors of 11βHSD1 in FF within the follicle (Thurston *et al.*, 2007).

1.4.7.1 Implications of 11βHSD modulators in COD

From all of the published findings described above, there is a chain of evidence that links cortisol and potentially 11βHSD1 activity to COD. Firstly, as previously mentioned in section 1.2.4 and 1.3.3.1, an increase in CRH, ACTH or glucocorticoid levels would be expected to inhibit the GnRH or LH pulse frequency and/or GnRH or LH release (Scholten and Liptrap, 1978; Dobson *et al.*, 1988; Williams *et al.*, 1990; Dobson *et al.*, 2000; Ribadu *et al.*, 2000). The lack of an LH surge prior to ovulation is associated with the formation of spontaneous ovarian cysts in COD. Glucocorticoids may also activate the sympathetic intraovarian nervous system and alter ovarian steroid production in pigs (Jana *et al.*, 2005). As glucocorticoids directly affect the HPA and HPG axes, and can additionally act on the ovary, these hormones, and therefore their metabolism catalysed by the 11βHSD enzymes, may be implicated in COD. Furthermore, ACRD, a human clinical syndrome with some similarity to COD, is characterised by an inability to regenerate cortisol, due to the impaired 11-KSR activity of 11βHSD1. However, as mentioned in 1.4.4, no mutations were found in the coding regions of the *HSD11B1* gene and it has also been documented that first degree male relatives of women with ACRD can regenerate cortisol (Jamieson *et*

al., 1999). This suggests that a post-translational effect of 11βHSD1 function, such as enzyme inhibition, may cause ACRD. Moreover, as males are unaffected, such enzyme inhibitors may be synthesised in females only. The intrafollicular inhibitors of 11βHSD1 found in the FF of women may thus be candidates in the pathogenesis of ACRD in women.

Interestingly, fluid from porcine and bovine spontaneous ovarian cysts exerted a more inhibitory effect on NADP(H)-dependent 11βHSD1 glucocorticoid metabolism than FF from large antral follicles (Thurston *et al.*, 2003b). Therefore, an alteration of ovarian glucocorticoid metabolism may be a cause, or consequence, of COD.

1.4.7.2 Hypothesis and objectives

In summary, details of 11βHSD1 and 11βHSD2 expression in ovarian follicle cells, the effects of glucocorticoids on various aspects of ovarian function, including steroidogenesis and oocyte maturation, and the occurrence of glucocorticoid inactivation in ovarian cells, all in a variety of species, have been presented in this chapter. Collectively, these findings indicate a role for glucocorticoid metabolism by the 11βHSD enzymes in mammalian folliculogenesis. In addition, the indirect effects of systemic glucocorticoids on LH secretion were suggested to be a cause of the formation of anovulatory follicles and potentially spontaneous ovarian cysts in COD. Higher levels of the intrafollicular 11βHSD1 inhibitors were present in the fluid from spontaneous porcine and bovine ovarian cysts than in FF from porcine and bovine preovulatory follicles. This raises the possibility that the endogenous enzyme modulators could be involved in the local modulation of ovarian 11βHSD1 activities. Furthermore altered levels of the 11βHSD1 inhibitors in ovarian fluids may be associated with, or contribute to, COD. As the intrafollicular modulators of 11βHSD1 were present in the ovarian fluids from pigs, cows and women, they may play roles in normal folliculogenesis and/or ovarian cyst formation in range of eutherian species.

The aim of the research present in this thesis was therefore to determine whether cortisol metabolism by the 11βHSD enzymes could play a role in ovarian folliculogenesis and/or COD. This overall aim was divided into five distinct experimental objectives:

1. To identify which isoforms of 11βHSD are expressed in the granulosa cells and COC of the porcine ovarian follicle.

2. To discover if 11βHSD activities change in granulosa cells and COCs with antral follicle growth.

3. To determine whether levels of intrafollicular modulators of 11βHSD1 are altered in FF during porcine antral follicle growth, compared to ovarian cysts.

4. To ascertain the effects of the modulators of 11βHSD1 in FF and cyst fluid on 11βHSD activities in rat kidney homogenates, and in porcine granulosa cells and COCs.

5. To establish the effects of the 11βHSD1 modulators in porcine ovarian fluids on the *in vitro* maturation (IVM) of porcine oocytes.

Chapter 2

General Materials and Methods

2.1 Chemicals and reagents

[1,2,6,7-^{3}H]-androst-4-ene-3,17-dione (androstenedione; specific activity = 80-110Ci/mmol), [1,2,6,7-^{3}H]-cortisol (69Ci/mmol), [1,2(n)-^{3}H]-cortisone (40Ci/mmol), [2,4,6,7-^{3}H]-oestradiol (81Ci/mmol) and [1,2,6,7-^{3}H]-progesterone (86Ci/mmol) were each purchased from GE Healthcare UK Ltd (Buckinghamshire, UK).

Agarose, foetal calf serum (FCS), L-glutamine, penicillin/streptomycin and Dulbecco's phosphate-buffered saline (DPBS) were provided from Life Technologies Ltd (Gibco BRL, Paisley, Strathclyde, UK).

Charcoal, chloroform (AnalaR® grade), di-sodium hydrogen orthophosphate 12-hydrate ($Na_2HPO_4.12H_2O$), ethanol (AnalaR® grade), ethyl acetate, gelatin (powder), methanol (AnalaR® grade), potassium chloride (KCl), sodium di-hydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$), thin-layer chromatography (TLC) aluminium sheets (20cmx20cm, Silica gel 60), trypan blue dye and ultra-pure water were supplied by BDH/Merck (Poole, Dorset, UK).

For the reverse transcription of total RNA, dNTP mix, dithiothreitol (DTT), 5× First-Strand Buffer, Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase, oligo $(dT)_{12-18}$ primer and Deoxyribonuclease I (Amplification grade), 10× PCR buffer, magnesium chloride ($MgCl_2$), dNTP mix and Platinum® *Taq* DNA polymerase were purchased from Invitrogen (Paisley, Strathclyde, UK).

Human long R3 IGF-1 was obtained from Gropep Limited (SA, Australia). Oestradiol antibody and antiserum were purchased from Guildhay Ltd (Guildford, Surrey, UK). Progesterone antibody and antiserum were supplied by the Central Veterinary Laboratory (Weybridge, Surrey, UK). Ultima-Gold scintillant was obtained from Packard Biosciences B.V (Pangbourne, Berkshire, UK).

Vectashield® Mounting Medium (H-1000) was purchased from Vector Laboratories (Burlingame, CA, USA).

All other chemicals, hormones or solvents not mentioned above were supplied by Sigma-Aldrich (Poole, Dorset, UK).

2.2 Collection of porcine ovarian samples

Porcine ovaries in the follicular phase of the ovarian cycle were obtained from an abattoir. Ovaries were transported to the laboratory in Medium 199 (M199) supplemented with 100IU/ml penicillin, 0.1mg/ml streptomycin, 2ml/L amphotericin B, 0.1% (w/v) BSA and 200nM L-glutamine at 25°C. In the laboratory, the ovaries were washed 3 times in warm sterile 0.9% (w/v) saline solution, then in 70% (v/v) ethanol for approximately 30 seconds before being rinsed in sterile saline.

Follicles of diameter 2–3mm (small antral follicles), 4–7mm (medium antral follicles) and ≥8mm (large antral follicles) (Knox, 2005) were dissected from porcine ovaries. All follicles were selected on the basis of a morphologically healthy appearance: follicles had a well-vascularised follicle wall and a translucent antrum containing no free-floating particles (Maxson *et al.*, 1985) (Guthrie *et al.*, 1995). Spontaneous ovarian cysts were dissected from cystic porcine ovaries; cysts were diagnosed as fluid-filled structures with diameters of 15–40mm on either ovary lacking CL (Heinonen *et al.*, 1998).

2.3 Aspiration and storage of porcine ovarian fluids

Samples of FF were aspirated from dissected small, medium and large antral follicles, and cyst fluid was drawn from ovarian cysts, using 21-gauge sterile needles. In order to generate sufficient quantities of each fluid (>1ml), samples of fluid from small and medium antral follicles were individually pooled from several follicles of the respective size category from an ovary. Fluids from large

antral follicles and single ovarian cysts were not pooled since single follicles/cysts each yielded >1ml of fluid. Each individual sample/pooled sample was aspirated from an ovary of one of 5 different animals. All fluid samples were centrifuged at 250 x g for 10 minutes at 4°C and the supernatant was aspirated leaving the cell pellet, including the oocyte, granulosa and theca cells, and other cell debris. In total, 5 pooled FF samples from small or medium antral follicles, 5 FF samples from large antral follicles and 5 cyst fluid samples were used in this study.

To confirm the visual assessment of follicles as being healthy/non-atretic, the intra-follicular oestradiol, androstenedione and progesterone concentrations were assessed as described in section 2.8, prior to the use of ovarian fluids in experimental studies.

2.4 Reverse phase C18 column chromatography of porcine ovarian fluids

C18 column chromatography was carried out as described (Thurston *et al.*, 2002; Thurston *et al.*, 2003b; Thurston *et al.*, 2003c). Firstly, C18 reverse phase chromatography mini-columns (Waters Chromatography, Hertfordshire, UK) were individually flushed with 10ml methanol and rinsed with 10ml distilled water (dH_2O). A 1ml volume of porcine FF or cyst fluid was then loaded on an individual C18 column. Fractions of fluid were sequentially eluted, based on hydrophobicity, by loading 1ml-volumes of increasing concentrations of methanol (0, 10, 20, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100% (v/v) methanol in dH_2O) through each C18 column. Each eluted fraction of ovarian fluid was collected into separate borosilicate glass tubes and evaporated to dryness at 45°C under nitrogen gas using a Techne Dri-Block DB-3 sample concentrator (Techne Ltd, Burlington, NJ, USA). Steroid residues from fractions eluted at 0 and 10% (v/v) methanol were re-suspended in 1ml of 0% (v/v) methanol and residues from fractions eluted at 20–100% (v/v) methanol were re-suspended in 1ml volumes of 20% (v/v) methanol. Each tube was stored at -20°C pending analysis.

2.5 Isolation of porcine granulosa cells

2.5.1 RT-PCR in porcine granulosa cells

After the aspiration and removal of ovarian fluids from dissected follicles or cysts, as detailed in section 2.3, the remaining shells from the small and medium antral follicles were dissected, and shells from large antral follicles and ovarian cyst shells were hemisected. Periantral and mural granulosa cells (collectively referred to as mural granulosa cells in the subsequent chapters of this thesis) were then gently flushed from the follicle shells using DPBS (leaving the basement membrane intact thereby preventing contamination with theca cells). The resultant cell preparations were washed by centrifugation in 15ml DPBS plus 2ml sterile water (to lyse erythrocytes) at 250 x *g* for 10 minutes at 4°C. The supernatant was then discarded and the cells were washed 3 times in 15ml DPBS at 250 x *g* for 10 minutes at 4°C to remove contaminating lymphocytes. Following these wash steps, the cell pellet was resuspended in 1ml DPBS and a 10µl aliquot was removed and mixed with 0.4% (w/v) trypan blue solution. To assess cell viability, the aliquot of cells in trypan blue was counted under a microscope (magnification x200) using a haemocytometer. Viable granulosa cells in the aliquot did not take up the trypan blue stain. Granulosa cell purity was estimated to be 95% in all cell cultures.

For each follicle category, RNA was extracted from 1 million viable cells according to the manufacturer's protocol for animal cells using the RNeasy Mini Kit (Qiagen Ltd, West Sussex, UK), which included an on-column DNase-treatment step to eliminate contaminating DNA. The same kit was used, as per the manufacturer's instructions, to extract total RNA from 30mg wet weight of porcine liver and kidney tissue, which acted as positive controls. RNA quantity and quality were assessed on an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA). The quality of RNA was indicated by an optical density (OD) 260:280 ratio of 1.8 to 2, and a 260:230 ratio of $\geq$1.8. 100-500ng RNA was incubated with 1µl 10mM dNTP mix, 2µl 0.1M DTT, 4µl 5× First Strand Buffer, 1µl 200U of M-MLV Reverse Transcriptase, 0.5µg oligo $(dT)_{12\text{-}18}$ primer and DNase/RNase-free water to give a

final volume of 20µl, before incubation at 37°C for 1 hour. RT(-) negative controls were also generated for all samples, in which RNA was incubated with DNase/RNase-free water in place of reverse transcriptase enzyme. The reaction was terminated by heating tubes at 95°C for 5 minutes before immediate placing on ice. The cDNA products were stored at -20°C prior to PCR amplification.

The primer sequences for porcine *hsd11b1* and *hsd11b2* were designed on the advice of Dr. Rob Fowkes and Miss Vicky Sharp using Primer3 online software (v 0.3.0) as described (Sharp *et al.*, 2007) Table 2.1 shows the forward and reverse primer sequences for each named primer, and the expected sizes of the PCR products. The nucleotide sequences, from which porcine primers were designed, were deposited on Genbank (accession numbers NM 214248 and NM 213913 for *hsd11b1* and *hsd11b2*, respectively). Primers for *hsd11b1* and *hsd11b2* had previously been validated to confirm primer specificity through the dideoxy-DNA sequencing of PCR products initially amplified from pig kidney, liver and testis cDNA (Sharp *et al.*, 2007). Levels of 18S ribosomal ribonucleic acid (rRNA) were used to confirm the integrity of the RT-PCR reaction, and oligonucleotide primers (accession number M10098) were designed from sequences known to be fully conserved between the human, rat, mouse and rabbit. Primers for porcine β-actin (Barboni *et al.*, 2000) and GAPDH (Zhu *et al.*, 2004) that had previously been published were run with each cDNA sample to further validate the reliability of the RT-PCR assay.

PCR reactions contained 2µl cDNA, 5µl 10× PCR buffer (200mM Tris-HCl, 200mM KCl), 1.5µl 50mM $MgCl_2$, 1µl 10mM dNTP mix, 1–1.5units Platinum® *Taq* DNA polymerase and 1µl 40µM forward and reverse outer primers for each primer set; reaction mixes were made up to 50µl with DNase/RNase-free water. Negative controls were prepared for each individual primer set using DNase/RNase-free water in place of cDNA (W). After initial denaturation at 94°C for 5 minutes on a Px2 Thermal Cycler (Thermo Electro Corporation, Waltham, MA, USA), 35 cycles of the following cycling conditions were used: denaturation at 94°C for 30 seconds, annealing at 55°C for each primer pair for 1 minute,

extension at 72°C for 1 minute and a final extension step at 72°C over 10 minutes. Each RT-PCR product was viewed after electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide and photographed under UV light, using the ImageQuant 400 (GE Healthcare, Amersham, Buckinghamshire, UK).

Table 2.1. Primer sequences, the expected product size and annealing temperatures for PCR.

Primer	Sequence	Product Size
11βHSD1	Forward 5'-CGCTCTGTATCCTCGGTCTC-3'	394 bp
	Reverse 5'-GTGTAGCGTAGAGTGTTCGA-3'	
11βHSD2	Forward 5'-CCAGCAGGAGATATGCCATT-3'	221 bp
	Reverse 5'-CTCGACGATGTCCGGATACC-3'	
18S	Forward 5'-CGATGCTCTTAGCTGAGTGT-3'	315 bp
	Reverse 5'-AGTCTATGGCAGCATCAAGG-3'	
β-actin	Forward 5'-ATCGTGCGGGACATCAAGGA-3'	169 bp
	Reverse 5'-AGGAAGGAGGGCTGGAAGAG-3'	
GADPH	Forward 5'-GGGCATGAACCATGAGAAGT -3'	162 bp
	Reverse 5'-GTCTTCTGGGTGGCAGTGAT-3'	

2.5.2 11βHSD dehydrogenase and reductase activities in porcine granulosa cells

Granulosa cells were isolated from small, medium and large antral follicles and from spontaneous ovarian cysts as described in section 2.5.1. After determining the number of viable isolated cells, cell pellets were re-suspended in serum-free McCoy's 5A medium supplemented with 100IU/ml penicillin, streptomycin (0.1mg/ml), bovine insulin (10ng/ml), human long R3 IGF-1 (10ng/ml), bovine transferrin (5μg/ml), sodium selenite (0.04ng/ml), androstenedione (100ng/ml)

and porcine FSH (1ng/ml). 5 x 10^4 viable cells/ml medium were seeded into triplicate sets of wells in a 24-well culture plate and incubated in a humidified atmosphere of 5% (v/v) CO_2 in air at 37°C for 24 hours. For the initial 20 hours of culture, cells were allowed to recover from any physical or functional injury during isolation from the ovary. During the last 4 hours of the 24-hour culture period, the net 11βHSD activities in the intact cells were assessed using the radiometric conversion assay as previously described by Thurston *et al.* (2002) and detailed below.

Net 11β-DH activities were assessed for 4 hours in triplicate sets of wells, each of which contained granulosa cells incubated in 900µl serum-free culture medium plus 100µl fresh serum-free medium containing 0.5µCi [1,2,6,7-^{3}H]-cortisol. Prior to addition to cell cultures, a solution of non-radioactive cortisol in culture medium was used to pre-dilute the [1,2,6,7-^{3}H]-cortisol (specific activity = 69Ci/mmol) to give the cortisol a final specific activity of 0.5µCi/pmol, and a final steroid concentration of 100pmol/ml (i.e. 100nM) in each well. Background cortisol oxidation was assessed in triplicate wells (acting as assay blanks) containing 1ml 0.1% (w/v) BSA in culture medium (in place of the granulosa cells) and cortisol, for comparison with net 11β-DH activities in granulosa cells.

Assays of net 11-KSR activity were measured in triplicate wells, each of which contained granulosa cells incubated in 900µl culture medium plus 100µl medium containing 0.1µCi [1,2(n)-^{3}H]-cortisone. Before addition to cells, the cortisone (specific activity = 40Ci/mmol) was diluted with non-radioactive cortisone in medium to give a final specific activity of 0.1µCi/pmol in each well, and a final cortisone concentration of 100nM. Background cortisone reduction was assessed in triplicate wells containing 1ml 0.1% (w/v) BSA in culture medium and cortisone, for comparison with net 11-KSR activities in granulosa cells.

Following the 4-hour incubation of cells (or assay blanks) with either radiolabelled cortisol or cortisone, the culture medium was removed from each well, transferred to Eppendorf tubes, and centrifuged at 250 x *g* for 10 minutes at

4°C to pellet the granulosa cells. After centrifugation, the supernatant was aspirated and transferred into triplicate sets of screw-cap borosilicate glass culture tubes, to which 2ml of ice-cold chloroform was added. The tubes were capped, vortexed and subsequently centrifuged at 1000 x *g* for 30 minutes at 4°C, after which two liquid phases could be observed in each sample tube. The upper, aqueous phase was removed and the lower, organic phase was evaporated to dryness at 45°C under nitrogen gas in a Techne Dri-Block DB-3 sample concentrator (Techne Ltd, USA), after which tubes could be stored at 4°C for up to 7 days. Steroid residues were then re-suspended in 30µl ethyl acetate containing 1mM non-radioactive cortisol and 1mM non-radioactive cortisone. The tubes were vortexed and 20µl from each tube was transferred to individual lanes of a TLC plate. TLC plates were resolved for approximately 90 minutes in TLC tanks containing 92:8 (v/v) chloroform:95% (v/v) ethanol. The bands containing cortisol and cortisone were visualised by absorption of UV light at 254nm. To complete the radiometric assay, the fractional conversion of [^{3}H]-cortisol to [^{3}H]-cortisone was assessed using a Bioscan System 200 radiochromatogramme scanner (Lablogic, Sheffield, UK), with a count time of 15 minutes per lane.

The final cortisol-cortisone inter-conversion was calculated by multiplication of percentage conversion of [1,2,6,7-^{3}H]-cortisol to [1,2,6,7-^{3}H]-cortisone (for 11β-DH activity) and [1,2,(n)^{3}H]-cortisone to [1,2,(n)-^{3}H]-cortisol (for 11-KSR activity) by the total amount of steroid that was present at the beginning of the assay (i.e. 100pmol/well). The respective 11βHSD activities in granulosa cells were calculated as net pmol of cortisone or cortisol produced per 5 x 10^4 viable cells over 4 hours for 11β-DH or 11-KSR activity, respectively (Michael *et al.*, 1997) (Thurston *et al.*, 2002; Thurston *et al.*, 2003c).

2.5.3 Cofactor-dependent 11βHSD activities in porcine granulosa cell homogenates

Granulosa cells from small, medium and large antral follicles and ovarian cysts were isolated, washed and counted as described in section 2.5.2. Each granulosa

cell pellet was then homogenised in a hypotonic lysis buffer consisting of 5mM Tris (pH 7.4) containing 1.5mM $MgCl_2$ and 1.5mM ethylenediaminetetraacetic acid (EDTA) (2.25ml lysis buffer/1x10^6 viable cells) (Thurston *et al.*, 2002), (Thurston *et al.*, 2003b),(Thurston *et al.*, 2003c). Isotonicity was restored to the cell homogenates by the addition of 10% (v/v) 1.5M KCl (0.25ml/1x10^6 viable cells). 100µl volumes of each homogenate were then transferred to triplicate sets of screw-cap borosilicate glass tubes containing 600µl DPBS. Triplicate tubes were prepared as assay blanks containing 600µl DPBS and 100µl 0.1% (w/v) BSA in DPBS in place of the ovarian cell homogenates, to assess background cortisol metabolism. Each triplicate set of tubes was pre-incubated for 30 minutes at 37°C in a gyratory waterbath. To assess net 11β-DH activities, each tube received 100µl DPBS containing either 4mM $NADP^+$ or NAD^+ cofactor, and 100µl DPBS containing 0.5µCi [^{3}H]-cortisol substrate (prepared as above to a final specific activity of 5Ci/mmol and a final cortisol concentration of 100nM). To determine net 11-KSR activities, each tube received 100µl DPBS containing 4mM NADPH cofactor ± 100µl DPBS supplemented with 10mM G-6-P, and 100µl DPBS containing 0.1µCi [^{3}H]-cortisone (final specific activity = 1Ci/mmol and final cortisone concentration = 100nM). After topping tubes up to a final volume of 1ml with DPBS, tubes were incubated in a gyratory waterbath for 4 hours at 37°C. Reactions were terminated by the addition of 2ml ice-cold chloroform to each tube. Radiolabelled steroids were then extracted and the radiometric conversion assay to quantify and calculate the net 11βHSD activities was completed as described in section 2.5.2.

2.6 Rat kidney homogenates

2.6.1 Preparation of rat kidney homogenates

Adult male Sprague-Dawley rats, which were housed and fed in accordance with the U.K. Animals (Scientific Procedures) Act 1986, were sacrificed by terminal anaesthesia with pentobarbitone sodium (Marks *et al.*, 2003). Kidneys were dissected and stored at -20°C. 0.5g rat kidney was added to 18ml hypotonic Tris-EDTA lysis buffer (buffer constituents listed in section 2.5.3), and homogenised

using a glass homogeniser. Isotonicity was restored with the addition of 2ml 1.5M KCl to yield a renal tissue homogenate concentration of 25mg/ml lysis buffer. The homogenate was centrifuged at 1000 x *g* for 20 minutes at 4°C, and the resulting supernatant was aspirated and retained for subsequent use in the assay.

2.6.2 Effects of porcine ovarian fluids and resolved fractions on $NADP^+$-dependent 11βHSD1 activity in rat kidney homogenates

From the homogenate supernatant, 100µl volumes were transferred to screw-cap glass tubes containing 600µl DPBS, as described in section 2.5.3. Triplicate tubes were prepared as assay blanks containing 600µl DPBS and 100µl BSA in DPBS (1mg/ml) in place of the renal tissue homogenates. All tubes were pre-incubated for 30 minutes at 37°C in a gyratory waterbath, as detailed above, before triplicate sets of tubes received either 100µl volumes of DPBS (controls and assay blanks), or 100µl FF from antral follicles or cyst fluid, or resolved fractions of FF or cyst fluid eluted at 0-100% (v/v) methanol. The 11β-DH activity of 11βHSD1 was assessed in renal homogenates by adding 100µl DPBS containing 4mM $NADP^+$ and 100µl DPBS containing 0.5µCi [^{3}H]-cortisol (pre-diluted to give a final specific activity of 5Ci/mmol and a final cortisol concentration of 100nM, as specified in section 2.5.3). Tubes were incubated at 37°C in a gyratory water bath for 1 hour, after which steroids were extracted and 11βHSD1 activities were measured as described in section 2.5.2.

2.7 Porcine cumulus-oocyte complexes (COCs)

2.7.1 Isolation of porcine COCs and oocytes

Samples of FF were aspirated from small, medium and large antral follicles using the protocol in section 2.3. Compact COCs were freshly isolated from the FF from small and medium antral follicles, and expanded COCs were collected from the FF from large antral follicles, under a Leica MZ125 light microscope (Leica Microsystems, Wetzlar, Germany) (magnification x20). Each type of COC was individually transferred into a dish containing M199 with 10% (v/v) FCS and kept

at 39°C. To remove all traces of FF from COCs, individual groups of freshly isolated compact or expanded COCs were rinsed twice in fresh M199 medium containing 10% (v/v) FCS, kept at 39°C. For assays involving the culture of denuded oocytes, individual groups of compact or expanded COCs were stripped of cumulus cells by vortexing in M199 containing 1% (w/v) hyaluronidase for 10 minutes. All traces of hyaluronidase were then removed by washing denuded oocytes with fresh M199 with 10% (v/v) FCS.

2.7.2 RT-PCR in compact porcine COCs

Compact COCs were collected from small and medium porcine antral follicles (as described in section 2.7.1) and pooled into DPBS containing 0.1% (w/v) BSA. Groups of 50-100 pooled compact COCs were then transferred into DNase/RNase-free sterile 0.5ml Eppendorf tubes containing 10µl lysis buffer (0.5% IGEPAL CA-630, 10mM Tris (pH 8.0), 10mM NaCl and 3mM $MgCl_2$. The lysed cells were then snap-frozen in liquid nitrogen and stored at -80°C prior to reverse transcription.

For the RT step, the lysates were first defrosted on ice and spun at 13,000 x *g* in a micro-centrifuge at 4°C for 1 minute. The extracted RNA was DNase-treated with Deoxyribonuclease I (Amplification grade) to eliminate contaminating genomic DNA, and then assessed for quantity and quality using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). 200–300ng RNA was reverse transcribed in a 20µl final reaction volume, with M-MLV Reverse Transcriptase, oligo(dT) primers, dNTP mix, DTT and 5× First Strand Buffer, as described in section 2.5.1, before incubation at 37°C for 1 hour. The reaction was terminated by heating tubes at 95°C for 5 minutes before immediately placing on ice. All cDNA produced was stored at -20°C until further use.

For the PCR step, the primers, PCR reaction constituents and PCR cycling conditions used were the same as described for granulosa cells in section 2.5.1. Porcine granulosa cells, and porcine liver and kidney tissue were used as positive

controls for all primer sets. Positive control primers run for each sample were 18S, β-actin and GAPDH. At the RT step, negative controls were prepared by incubating each cDNA sample without reverse transcriptase enzyme (RT-) and further negative controls were prepared at the PCR step by adding DNase/RNase-free water in place of cDNA for each individual primer set (W). Each RT-PCR product was viewed after electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide and photographed under UV light using the ImageQuant 400 (GE Healthcare, UK), as in section 2.5.1.

2.7.3 11βHSD dehydrogenase activities in porcine COCs and oocytes

Net 11βHSD activities were assessed in groups of 5 freshly isolated compact COCs or 5 denuded oocytes from compact COCs per well, or in single expanded COCs and single denuded oocytes from expanded COCs. Each group of COCs or denuded oocytes were seeded into triplicate sets of wells within 96-well culture plates and cultured for 24 hours in a humidified atmosphere of 5% (v/v) CO_2 in air at 39°C. Triplicate wells contained 90µl M199 medium with 10% (v/v) FCS, to which 10µl of [1,2,6,7-^{3}H]-cortisol (final specific activity = 5Ci/mmol) was added. Both radiolabelled substrates were pre-diluted as detailed in section 2.5.2. Background cortisol metabolism was assessed in triplicate wells containing 100µl 0.1% (w/v) BSA in M199 in place of the COCs or oocytes.

After 24 hours in culture, the medium from each well was aspirated, placed into individual 1.5ml Eppendorf tubes and centrifuged at 250 x *g* for 10 minutes at 4°C to pellet denuded oocytes or COCs. The supernatant medium, minus the COCs/oocytes, was then transferred into screw-cap glass tubes, after which the steroids were extracted into 2ml ice-cold chloroform, and net 11βHSD activities were assessed using TLC as described in section 2.5.2. The respective 11βHSD activities in COC or denuded oocytes were calculated as net pmol of cortisone produced per COC or oocyte over 24 hours.

2.7.4 *In vitro* maturation (IVM) of porcine compact COCs

Porcine compact COCs were isolated from small and medium antral follicles as described in 2.7.1. COCs were selected for the IVM study, under a Leica MZ125 microscope (Leica Microsystems, Germany) on the basis of having >1 uniform layer of cumulus cells surrounding the oocyte. Groups of 30 compact COCs were cultured in 200µl serum-supplemented M199 medium containing 100IU/ml penicillin, 0.1mg/ml streptomycin, 0.1% (w/v) BSA, 0.57mM cysteine, 3mM L-glutamine, 10ng/ml EGF, 500ng/ml FSH and 500ng/ml LH. Groups of COCs were also cultured in the above maturation medium containing 10% (v/v) cyst fluid, or resolved fractions of cyst fluid. Resolved fractions of cyst fluid eluted between 0-100% (v/v) methanol were generated as described in section 2.4. The use of resolved fractions in this study differed to prior studies, however, as fractions of cyst fluid eluted at 0-20% (v/v) methanol (hydrophilic fractions) were pooled together, as was the case for the fractions of fluid eluted at 70-100% (v/v) methanol (hydrophobic fractions). Individual groups of COCs were then either incubated with the pooled fractions of cyst fluid eluted at 0-20% (v/v) methanol, or the pooled fractions eluted at 70-100% (v/v) methanol. Each set of pooled fractions was reconstituted in 1ml 20% (v/v) methanol and tested at a final dilution of 10% (v/v), giving final methanol concentration of ≤2% (v/v) in all samples. Therefore, the control groups of oocytes were incubated in the absence of pooled fraction of cyst fluid but in the presence of 2% (v/v) methanol. COCs were cultured in 96-well plates for 48 hours in a humidified atmosphere of 5% (v/v) CO_2 in air at 39°C.

After 48 hours in culture, COCs were denuded as described in section 2.7.1, and the state of maturation was visually assessed using the Leica MZ125 microscope (Leica Microsystems, Germany), as above, by noting the presence or absence of a PB, indicating whether an oocyte had progressed to MII and undergone maturation, or not, respectively. The nuclear maturation of COCs was confirmed using fluorescence microscopy by staining the chormatin with propidium iodide (PI), and on staining the microtubules with a fluorescently-labelled α-tubulin antibody (detailed below in section 2.7.6).

2.7.5 Assessment of nuclear maturation state of porcine oocytes

After the 48-hour culture period, a proportion of COCs from each treatment group were denuded, as detailed above, and the chromatin and microtubules in certain oocytes were fluorescently stained for evidence of nuclear maturation. Groups of denuded oocytes were then fixed for 30mins at 37°C in 500µl DPBS containing 2.5% (v/v) paraformaldehyde and 0.5% (v/v) Triton X-100. After fixing, oocytes were washed three times in 500µl DPBS containing 2% (v/v) FCS and 0.5% (v/v) Triton X-100, before being incubated in blocking solution (500µl DPBS containing 20% (v/v) FCS and 0.5% (v/v) Triton X-100) for 30mins at 37°C. Oocytes were then transferred into primary antibody (mouse monoclonal α-tubulin antibody at a 1/200 dilution, 500µl per well) and incubated for 1 hour at 37°C, after which cells were washed three times, as above. Oocytes were then incubated in secondary antibody (fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin G antibody at a 1/400 dilution, 500µl per well) for 1 hour at 37°C. Cells were washed three times before being incubated with 500µl 10µg/ml PI for 10mins at 37°C to stain the chromatin. Finally, stained oocytes were mounted with Vectashield® Mounting Medium (Vector Laboratories, USA) to preserve fluorescence, using a raised coverslip. The staining of the oocytes was then visualised under an Olympus IX81 inverted microscope (Olympus Imaging, Tokyo, Japan) in bright field, and with FITC or tetramethylrhodamine isothiocyanate (TRITC) filters. Figures showing examples of each stage of oocyte maturation are presented in chapter 6.

2.7.6 11βHSD activities in porcine oocytes derived from IVM studies

At the end of the IVM culture period, fluorescence-staining of the oocytes lacking PBs indicated that no nuclear maturation had occurred (an example of which is shown in Figure 6.9a). On the contrary, a metaphase plate (an example of which is shown in Figure 6.9b) was observed in all oocytes exhibiting PBs that were selected for staining and visualised under an Olympus IX81 inverted microscope (Olympus Imaging, Tokyo, Japan).

Therefore, oocytes that had extruded a PB when viewed under a Leica MZ125 microscope (Leica Microsystems, Germany) were adjudged to have reached MII. Thus, after the IVM study described in section 2.7.5, oocytes not exhibiting a PB were separated from those that did, and divided into individual groups of 5. Net 11βHSD activities were then determined in each oocyte group, in triplicate, as described in section 2.7.3. Net enzyme activities in these IVM-derived oocytes were assessed alongside activities in denuded oocytes from freshly isolated compact COCs that had been collected from porcine ovaries collected from the abattoir on the same morning.

2.8 Measurement of hormone concentrations

2.8.1 Determination of intra-follicular progesterone concentrations using radioimmunoassay (RIA)

Progesterone concentrations were determined in FF from small, medium and large antral follicles, and in cyst fluid, by RIA as previously described by Pallikaros *et al.* (1995). A progesterone standard curve was prepared from a 1μg/ml stock solution of progesterone in ethanol, stored at -20°C. A volume of 10μl was removed from the progesterone standard, and the ethanol was evaporated under nitrogen gas. The progesterone was re-suspended in 1ml serum-free medium to produce the top standard progesterone concentration of 10ng/ml (31.797nM). The top standard was double-diluted in medium to produce the following standards: 15.898, 7.949, 3.975, 1.987, 0.994, 0.497, and 0.248nM progesterone. Volumes of 100μl were aliquoted in triplicate and 100μl serum-free M199 medium was aliquoted into the 0nM progesterone (zero standard; B_0), total binding and non-specific binding tubes in triplicate. The unknown samples were serially diluted in serum-free medium into a total volume of 100μl. The unknown samples were diluted between 1/100 to 1/200 into a total volume of 100μl per tube.

Phosphate azide saline-gelatin (PAS-Gel) buffer was used to dilute progesterone antiserum. PAS-gel buffer was made by dissolving 10 PBS tablets and 0.1g sodium azide in 1 litre of dH_2O, before dissolving 1g gelatin whilst stirring the

mixture over a hotplate. Progesterone antiserum was diluted 1/100 (v/v) in PAS-Gel buffer and stored in 100µl aliquots at -20°C. Immediately prior to use, a 100µl aliquot was thawed and further diluted 1/100 (v/v) in PAS-Gel buffer resulting in a working antibody dilution of 1/10,000 (v/v). A 100µl aliquot of diluted anti-progesterone antibody was added to each standard and sample tube, excluding those for the determination of radioactivity in total binding and non-specific binding tubes, in which 100µl of PAS-Gel buffer was substituted. [1,2,6,7-^{3}H]-Progesterone was diluted in PAS-Gel buffer to give the required 10,000cpm per 100µl. A volume of 100µl diluted [1,2,6,7-^{3}H]-progesterone was added to all assay tubes. Each tube was vortexed, covered in aluminium foil, and stored at 4°C overnight. While assay tubes were incubating, a solution of 0.025% (w/v) dextran was prepared in PAS-Gel buffer. After the dextran had dissolved, 0.25% (w/v) charcoal was added and mixed for 30 minutes, after which the solution was kept at 4°C overnight.

The following day, a volume of 500µl activated dextran-coated charcoal was added to each assay tube, with the exception of the total binding tubes, which received 500µl PAS-Gel buffer. The tubes were vortexed and centrifuged for 10 minutes at 1000 x *g* at 4°C with no brake to precipitate unbound progesterone. The supernatants were decanted into mini-scintillation vials, and 2ml Ultima-Gold scintillant was added into each vial, which were subsequently capped and vortexed. Radioactivity in cpm was measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter (Beckman Coulter, Fullerton, CA, USA), with a count time of 1 minute per vial. The counts were analysed using a DOS-RIA software program using the logit % B/B_0 whereby the data from the standard curve was linearised. There was a detection limit of 0.5nM progesterone for this RIA method, with inter- and intra-assay coefficients of variation of 8.9% (n = 15) and 13.6% (n = 40), respectively, at 31% binding.

2.8.2 Measurement of intra-follicular androstenedione concentrations

Intra-follicular concentrations of androstenedione were determined in FF from small, medium and large antral follicles, and in cyst fluid, by RIA, as previously described (Wrathall and Knight, 1995). As for the progesterone RIA described in 2.8.1, a standard curve was set up by double-dilution of the top androstenedione standard of 5.00nM, giving further standards consisting of 2.50, 1.00, 0.50, 0.25, 0.10 and 0.05nM androstenedione. The zero standards, total binding and non-specific binding tubes were prepared as described in 2.8.1 and again, androstenedione antiserum and the tritiated hormone were diluted in PAS-Gel buffer. Unknown samples were diluted to 1/100 and 1/400 into a total volume of 100µl per tube. Following addition of the appropriate contents of each assay tube, every tube was vortexed, covered in aluminium foil and kept overnight at 4°C. A solution of dextran-coated charcoal was also made up and stored at 4°C overnight. On the next day the protocol described in 2.8.1 was followed to assess the radioactivity in each tube. There was a detection limit for this RIA method of 50pM androstenedione, with inter- and intra-assay coefficients of variation of 10% and 8%, respectively.

2.8.3 Determination of intra-follicular oestradiol concentrations by ELISA

Intra-follicular oestradiol concentrations in FF from small, medium and large antral follicles, and in cyst fluid, were measured by enzyme-linked immunosorbent assay (ELISA) using a kit (EIA-2693) purchased from DRG Diagnostics (Marburg, Germany). The unknown samples were diluted to 1/100 and 1/400, into a total volume of 25µl per well, and these were incubated for two hours at room temperature. The unknown oestradiol concentration in the sample competes with a fixed amount of oestradiol conjugated with horse-radish peroxidase for the binding sites of a polyclonal oestradiol antiserum coated onto each well. The final concentration of oestradiol in the unknown sample is inversely related to the optical density measured by a microwell reader. Intra-follicular concentrations were determined from optical density data using the DRG ELIZA MAT 2000 and the DRG Regression Program. The detection limit

of this ELISA was 5pM oestradiol, with intra- and inter-assay coefficients of variation of 5% and 2%, respectively.

2.8.4 Measurement of oestradiol production by porcine granulosa cells using RIA

This RIA was conducted as previously described (Mason *et al.*, 1994). An oestradiol standard curve was prepared from a top standard concentration of 9.180nM oestradiol, which was double-diluted in medium to produce the following standards: 4.590, 2.295, 1.148, 0.574, 0.287, 0.143, 0.072 and 0.036nM oestradiol. Volumes of 200µl of each oestradiol standard were aliquoted in duplicate, and 200µl serum-free M199 medium was aliquoted into the 0nM oestradiol (zero standard), total binding and non-specific binding tubes in duplicate. Unknown samples were comprised of the medium aspirated from each well of the granulosa cell culture, as first described in section 2.5.2. A volume of 200µl for each unknown medium sample was added into the assay tube, either neat or at a dilution of 1/10 with medium, with a total final volume of 400µl per assay tube. PAS-gel buffer was used for the dilution of oestradiol antiserum and antibody as described in 2.8.1. The various assay tubes were set up, incubated and the radioactivity was counted as described above. There was a detection limit of 2.4pM oestradiol for this RIA, with inter- and intra-assay coefficients of variation were 6% and 10%, respectively. The oestradiol antibody exhibited 12% cross-reactivity with oestrone, 1.3% cross-reactivity with oestriol and <0.1% cross-reactivity with testosterone, progesterone and cortisol.

2.8.5 Measurement of total intra-follicular cortisol and cortisone by RIA

Total intra-follicular cortisol and cortisone concentrations (i.e. free plus protein-bound steroid concentrations) in FF from small, medium or large antral follicles, and in cyst fluid, were measured using a previously described (Moore *et al.*, 1985; Wood *et al.*, 1996). The RIA for cortisol had a range of 30–2000nM with intra- and inter-assay coefficients of variation of <7% and <8% respectively. The RIA for cortisone had a range of 4–500nM with intra- and inter-assay coefficients of

variation of <8% and <10% respectively. The cortisol antibody exhibits <1.2% cross-reactivity with cortisone, and there is <0.1% cross-reactivity between the cortisone antibody and cortisol, and both anti-cortisol and anti-cortisone antibodies exhibited <0.1% cross-reactivity with progesterone and <1% with 17α-hydroxyprogesterone.

2.9 Statistical Analysis

To assess whether data were normally distributed, the Kolmogorov-Smirnov (KS) test was employed and the majority of data were then compared using one-way ANOVA followed by either Tukey's or Dunnett's multiple comparison as the *post hoc* test. The correlation between the intra-follicular progesterone concentration in FF from small, medium and large antral follicles and porcine cyst fluid and the effects of those same fluid samples on 11βHSD1 activity in rat kidney homogenates was calculated as the Pearson's correlation coefficient (Figure 4.7). For the experiment where exogenous cofactors were added to granulosa cell homogenates (Figure 3.4), and where FF and cyst fluid were added to cultures of intact granulosa cells (Figure 5.1), comparisons between conditions were restricted to within a given follicle size category. To analyse statistical differences between 11βHSD activities in oocytes that did not or did extrude PBs after IVM, a two-tailed, unpaired T test with Welch's correction (for unequal variances) was performed. The chi-squared test was employed to analyse data from IVM studies, to compare the proportion of oocytes that had or had not extruded a PB in the presence or absence of cyst fluid (Table 6.1), or resolved fractions thereof (Table 6.2). All statistical evaluations were performed on absolute, non-referenced data using GraphPad Prism4 software (San Diego, CA, USA) (although selected data are presented graphically as the percentage of control enzyme activities in the absence of treatments). In all cases, values of $P<0.05$ were accepted to indicate statistical significance.

Chapter 3

11βHSD Expression and Activities in Porcine Granulosa Cells

3.1 Background

The conversion of cortisol to cortisone in human granulosa cells was first measured by Owen *et al.* (1992), although Michael *et al.* (1993a) were the first to attribute this oxidation to 11βHSD. It was later established that there is almost exclusive net 11β-DH activity in human granulosa cells isolated from small antral follicles. Levels of net cortisol oxidation were similar in human granulosa cells from small and large antral follicles, however, 11-KSR activities were significantly increased in the granulosa cells from preovulatory follicles (Yong *et al.*, 2000).

Tetsuka *et al.* (1997) were the first to demonstrate exclusive 11βHSD2 expression in human follicular granulosa cells and exclusive 11βHSD1 expression in luteinising human granulosa cells. This change of 11βHSD isoform expression at ovulation would appear to correlate with the afore-mentioned changes in net activities of 11βHSD in granulosa cells from human small antral and preovulatory follicles (Yong *et al.*, 2000). The almost exclusive net cortisol oxidation observed in granulosa cells from small antral follicles is presumably catalysed by 11βHSD2, whereas the significantly higher reductase activities in granulosa cells from preovulatory follicles may be reflective of an increase in 11βHSD1 expression prior to ovulation. As mentioned in section 1.4.6, the absolute switch in 11βHSD isoform expression in granulosa cells across the ovarian cycle has been demonstrated in human (Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Ricketts *et al.*, 1998) and rat granulosa cells (Tetsuka *et al.*, 1999a; Tetsuka *et al.*, 1999b). In the cow ovary, however, both cloned 11βHSD enzymes are co-expressed in granulosa cells during both phases of the ovarian cycle, with 11βHSD2 expression dominant in follicular granulosa cells and predominant 11βHSD1 expression in the active CL (Tetsuka *et al.*, 2003; Thurston *et al.*, 2007). To date no 11βHSD enzyme expression or activity studies have been carried out in the pig ovary.

The aims of this study were therefore:

1. To identify which 11βHSD enzymes are expressed in porcine granulosa cells from small, medium and large antral follicles, as well as from spontaneous ovarian cysts.

2. To measure 11βHSD activities (11β-DH and 11-KSR) in porcine granulosa cells from antral follicles and ovarian cysts, and determine whether enzyme activities differ with the stage of follicle growth.

3. To determine the cofactor preference of the 11βHSD enzymes in granulosa cells from antral follicles and ovarian cysts.

4. To determine the effects of FSH on 11βHSD activities in granulosa cells.

3.2 Results

3.2.1 Expression of the 11βHSD enzymes in granulosa cells from porcine antral follicles

RT-PCR assays, which were conducted as described in section 2.5.1, required the initial extraction of total RNA from the granulosa cells. It was not possible to conduct RT-PCR assays in granulosa cells from porcine ovarian cysts, however, due to a technical difficulty in extracting sufficient quantities of intact total RNA from granulosa cells of ovarian cysts. This may have been due to the rapid enzymatic degradation of RNA in granulosa cells from ovarian cysts.

Porcine liver and kidney were used as positive control tissues for all primer sets. Positive control primers run for each sample were 18S, β-actin and GAPDH. At the RT step, negative controls were prepared by incubating each total RNA sample without reverse transcriptase enzyme (RT-) and additional negative controls were prepared at the PCR step by adding DNase/RNase-free water in place of cDNA for each individual primer set (W).

Figure 3.1 depicts a representative RT-PCR product gel which shows the cell- or tissue-specific 11βHSD1 or 11βHSD2 mRNA expression that was demonstrated in 3 individual sows. Primers designed for *hsd11b1* amplified cDNA in porcine granulosa cells from small, medium and large antral follicles and from porcine liver. The *hsd11b2* primers amplified cDNA in porcine granulosa cells from small, medium and large antral follicles and from porcine kidney. Negative control RT(-) samples, incubated with *hsd11b1* or *hsd11b2* primers, showed that no genomic DNA was present in any of the cell or tissue samples. The negative water control indicated that the PCR equipment or reagents were not contaminated with any nucleic acids.

Figure 3.2 shows a representative RT-PCR product gel depicting the cell- or tissue-specific 18S, β-actin or GAPDH mRNA expression in 3 individual sows. Bands resulting from the amplification of cDNAs encoding 18S, β-actin and GAPDH were identified in all cells/tissues on all gels. The detection of amplicons for 18S, β-actin and GAPDH confirmed the integrity of the RNA isolated from all cell and tissue samples, and the detection of β-actin and GAPDH products indicated that the RT step had been successful for cell/tissue samples. Negative control RT(-) samples were run for all positive control primers in all cell/tissue samples and confirmed the absence of genomic DNA in all samples (these have not been shown on this gel). The negative water control indicated that the PCR equipment or reagents were not contaminated with any nucleic acids.

3.2.2 11βHSD activities in granulosa cells from porcine antral follicles and ovarian cysts

Basal 11β-DH and 11-KSR activities of 11βHSD were measured in porcine granulosa cells from small, medium and large antral follicles and ovarian cysts, as described in section 2.5.2. In brief, 5 x 10^4 viable granulosa cells/ml medium were seeded into each well of a 24-well plate and cultured for a total of 24 hours. For the first 20 hours, granulosa cells were allowed to recover from the physical process of cell isolation from the ovary. After this time, enzyme substrate was added and a radiometric conversion assay was conducted over 4 hours in order to

measure enzyme activity. To assess net 11β-DH activities, cultured granulosa cells received radiolabelled plus non-radiolabelled cortisol with a final specific activity of 0.5μCi/100pmol at a final concentration of 100pmol/ml. To measure net 11-KSR activities, cells were incubated with radiolabelled plus non-radiolabelled cortisone at a final specific activity of 0.1μCi/100pmol and a final concentration of 100pmol/ml. After a 4-hour incubation with either substrate, ^{3}H-steroids were resolved and quantified by TLC to determine net 11β-DH or 11-KSR activities of the 11βHSD enzymes. No 11-KSR activities were detected in granulosa cells from antral follicles, irrespective of follicle size, or from ovarian cysts. Consequently, only changes in net 11β-DH activities will be described hereafter.

Net 11β-DH activities were lowest in granulosa cells from small antral follicles and were significantly increased, by approximately 3-fold, in granulosa cells from large antral follicles ($P<0.05$; Figure 3.3). Levels of net cortisol oxidation in cells from ovarian cysts were comparable to those displayed in small antral follicles and were significantly lower than enzyme activities in granulosa cells from large antral follicles ($P<0.05$).

3.2.3 Effects of cofactor addition on 11βHSD activities in granulosa cell homogenates from porcine antral follicles and ovarian cysts

As described in section 2.5.3, homogenates were prepared from granulosa cells isolated from antral follicles and ovarian cysts. Cell homogenates were then incubated for 4 hours under different cofactor conditions: with $NADP^{+}$ or NAD^{+} to measure 11β-DH activities, and with NADPH in the presence or absence of 10mM glucose-6-phosphate (G-6-P) to measure the 11-KSR activities of 11βHSD1. G-6-P was added with exogenous NADPH to certain cell homogenates to enhance the co-operative activity of H6PDH and 11βHSD1 in the lumen of the ER. As discussed in section 1.4.2.2, H6PDH catalyses the first two reactions of the pentose phosphate pathway, including the conversion of G-6-P to 6-phosphogluconate, which can generate NADPH to drive the 11-KSR activity of

11βHSD1 (Draper *et al.*, 2003; Atanasov *et al.*, 2004; Bujalska *et al.*, 2005; Czegle *et al.*, 2006; Odermatt *et al.*, 2006).

As for the study in cultured granulosa cells described in section 3.2.1, no 11-KSR activities were detected in granulosa cell homogenates from any antral follicles or ovarian cysts subsequent to incubation with NADPH, either with or without G-6-P. Therefore, only the effects of cofactor addition on the $NADP^+$- and NAD^+-dependent activities of the 11βHSD enzymes will be discussed.

Irrespective of follicle size, cortisol oxidation was low (≤0.2pmol cortisone/4h) in granulosa cell homogenates incubated without exogenous cofactor (Figure 3.4). For each follicle category, both the $NADP^+$- and NAD^+-dependent activities of 11βHSD were higher than activities in the respective granulosa cell homogenates lacking exogenous cofactors. There were no significant differences between $NADP^+$- and NAD^+-dependent enzyme activities in granulosa cells, irrespective of the follicle category from which cells were homogenised.

In porcine antral follicles, both $NADP^+$- and NAD^+-dependent cortisol oxidation in granulosa cell homogenates increased with antral follicle diameter. $NADP^+$-dependent 11βHSD activities increased successively by approximately 3-fold, from 0.5±0.1pmol cortisone/4h in homogenates from small antral follicles to 1.2±0.3pmol cortisone/4h in large antral follicles. Levels of NAD^+-dependent cortisol oxidation in granulosa cells from large antral follicles (1.0±0.2pmol cortisone/4h) were 2-fold higher than in small antral follicles (0.5±0.1pmol cortisone/4h). In granulosa cell homogenates from ovarian cysts, $NADP^+$-dependent enzyme activities were comparable to those measured in homogenates from large antral follicles whereas NAD^+-dependent 11βHSD activities in granulosa cell homogenates from ovarian cysts were similar to the respective enzyme activities in cell homogenates from small and medium antral follicles.

3.2.4 Effects of FSH on 11βHSD activities in granulosa cells from porcine large antral follicles

In this study, granulosa cells from large antral follicles were cultured, as detailed in section 2.5.2, and 11βHSD activities were measured in the presence of 0-100ng/ml FSH. Since no 11-KSR activities were detected in granulosa cells or cell homogenates from antral follicles or ovarian cysts (as described in sections 3.2.2 and 3.2.3), only effects of FSH on net 11β-DH activities were assessed. In brief, 5 x 10^4 viable granulosa cells/ml medium were seeded into each well of a 24-well plate and after 20 hours in culture, a 4-hour radiometric conversion assay was carried out in the presence of a range of concentrations of FSH (0, 0.1, 1, 10 and 100ng/ml). 11βHSD activities measured in the absence of FSH (0.9±0.2pmol cortisone/4h) showed no variation from any activities measured in the presence of FSH, irrespective of FSH concentration ($P>0.05$; Figure 3.5).

3.2.5 Effects of FSH on oestradiol production in granulosa cells from porcine large antral follicles

To validate the concentrations of FSH used to assess potential effects of this gonadotrophin on net cortisol oxidation by the 11βHSD enzymes in granulosa cells, the same range of FSH concentrations (0-100ng/ml) were assessed for effects on oestradiol production by the granulosa cells. After a 24-hour incubation period, the culture medium was aspirated from each well and oestradiol concentrations were determined by RIA (see section 2.8.4). Basal oestradiol production (0.7pmol oestradiol/24h) was not altered in the presence of increasing concentrations of FSH ($P>0.05$; Figure 3.6).

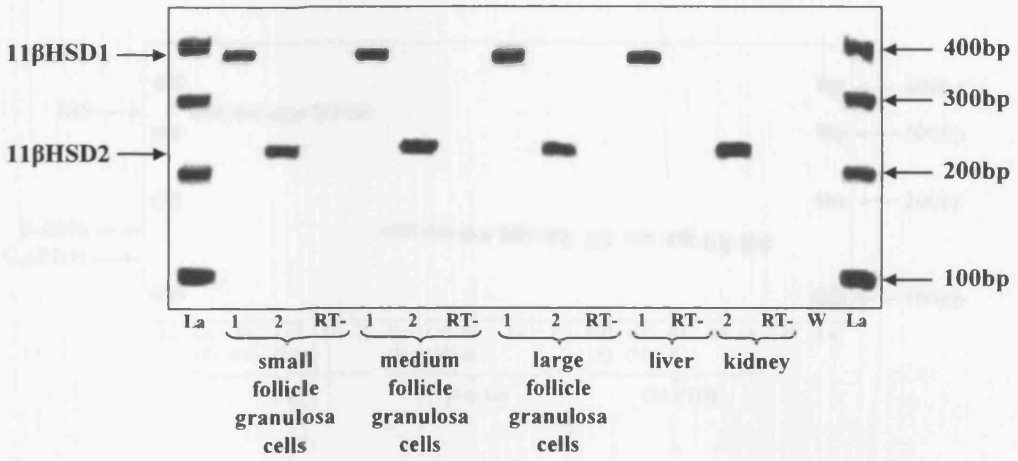


Figure 3.1. 11βHSD1 and 11βHSD2 mRNA expression in porcine granulosa cells, and porcine liver and kidney. Amplicons generated for 11βHSD1 and 11βHSD2 cDNA were of the predicted sizes (394bp and 221bp, respectively). Gel lanes are marked as follows: La = ladder, then 11βHSD1 (1), 11βHSD2 (2) and RT(-) amplicons for granulosa cells from small, medium and large antral follicles, 11βHSD1 and RT(-) amplicons for liver tissue, 11βHSD2 and RT(-) samples for kidney tissue. RT(-) samples were incubated with DNase/RNase-free water in the place of reverse transcriptase. W = water control, in which DNase/RNase-free water was amplified in place of cDNA.

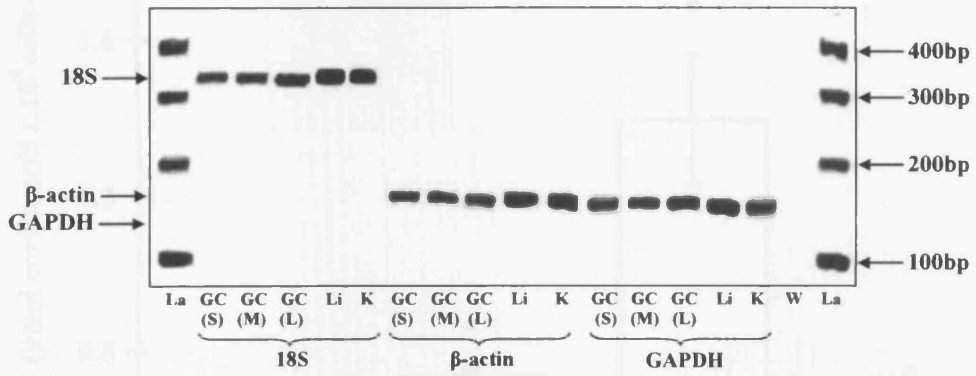


Figure 3.2. 18S, β-actin and GAPDH expression in porcine granulosa cells, and porcine liver and kidney. Amplicons generated for 18S, β-actin and GAPDH were of the predicted sizes (315bp, 169bp and 162bp respectively). Gel lanes are marked as follows: La = ladder, GC(S), GC(M) and GC(L) = granulosa cells from small, medium and large antral follicles, respectively, Li = liver tissue, K = kidney tissue and W = water control. In the water control, DNase/RNase-free water was amplified in place of cDNA.

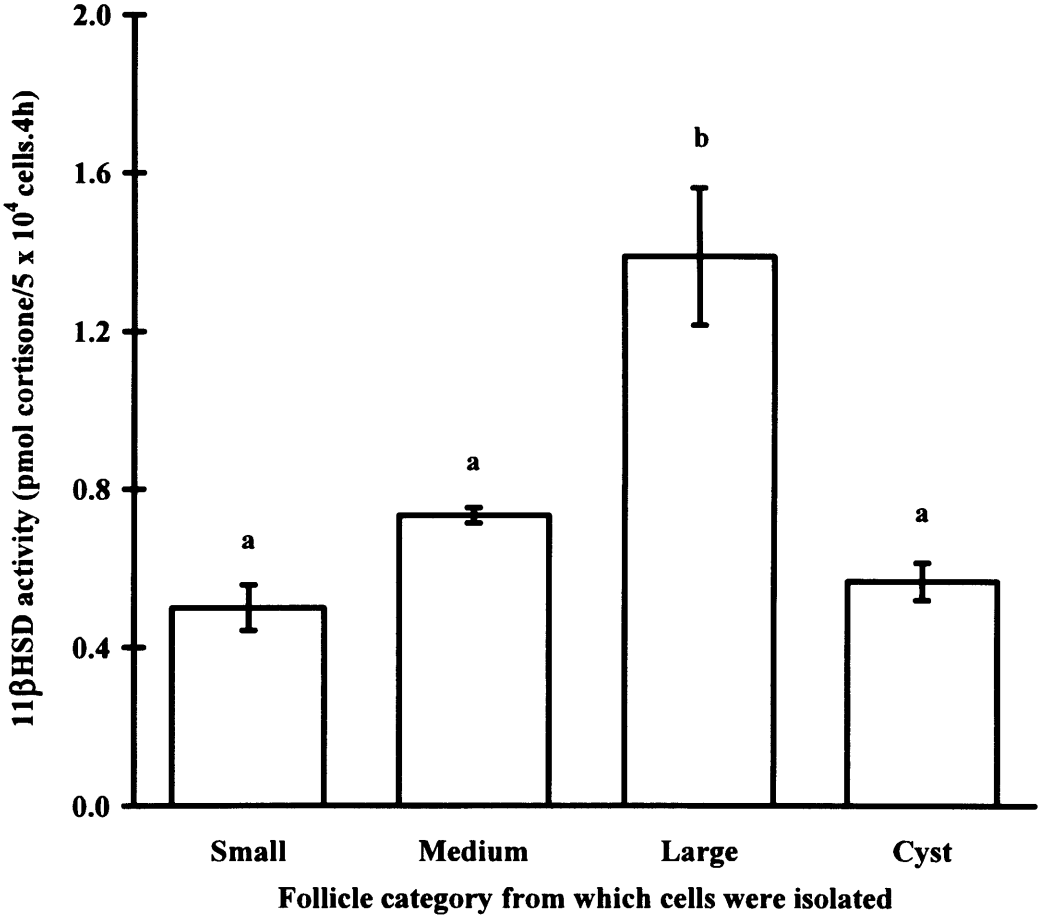


Figure 3.3. Net cortisol oxidation in porcine granulosa cells isolated from small, medium and large antral follicles and from spontaneous ovarian cysts. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone/5 x 10^4 viable cells.4h) for 5 independent granulosa cell cultures from antral follicles or ovarian cysts from different animals. Between bars, data showing different superscripts differ significantly ($P<0.05$).

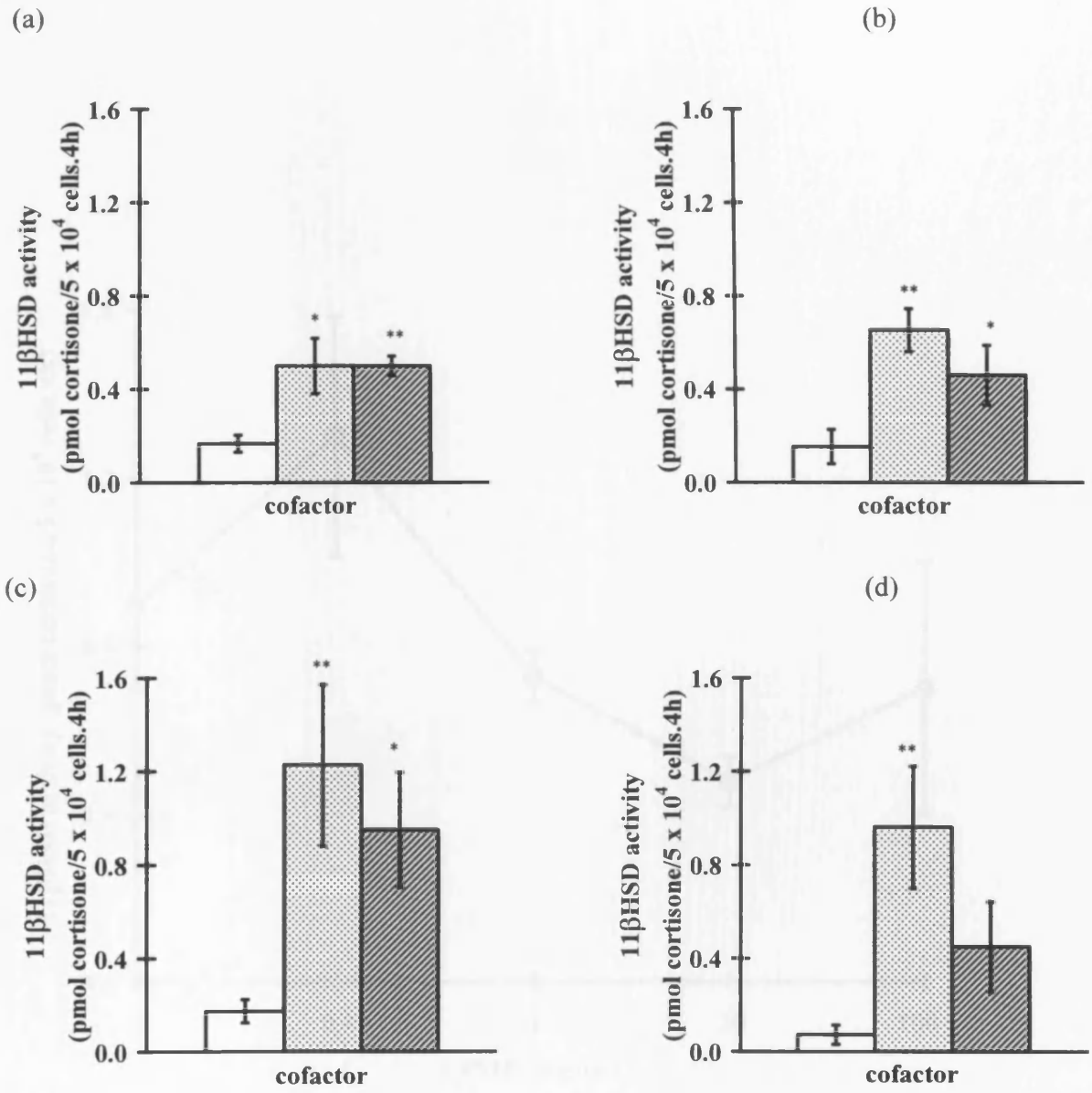


Figure 3.4. Net cortisol oxidation, with and without exogenous cofactor addition, in homogenates of porcine granulosa cells isolated from (a) small, (b) medium and (c) large antral follicles and (d) spontaneous ovarian cysts. Each measurement represents the mean (±SEM) enzyme activity (pmol cortisone/5 x 10^4 viable cells.4h) for 5 independent assays of granulosa cell homogenates from antral follicles or ovarian cysts, from different animals, in the absence of cofactors (open bars), in the presence of 4mM $NADP^+$ (stipled bar) or in the presence of 4mM NAD^+ (hatched bar). *P<0.05 and **P<0.01 versus the respective rate of cortisol oxidation measured in the absence of cofactors.

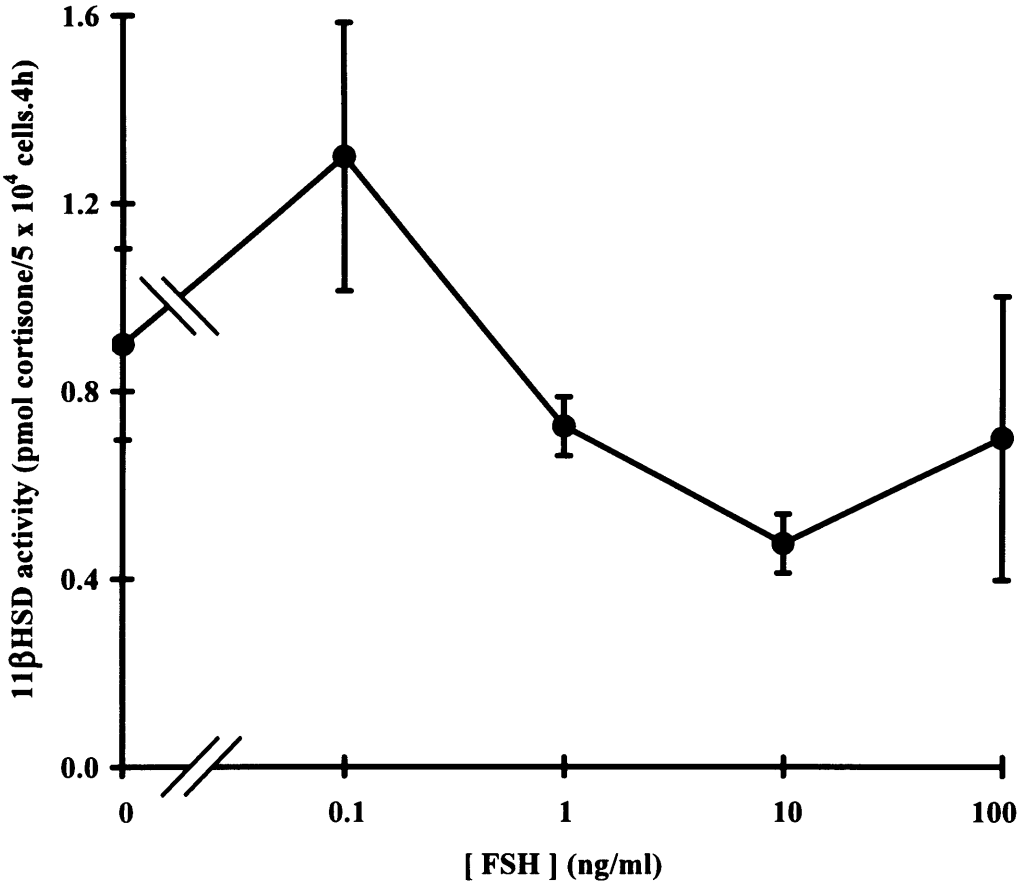


Figure 3.5. Effects of FSH on net cortisol oxidation in porcine granulosa cells isolated from large antral follicles. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone/5 x 10^4 viable cells.4h) for 3 independent granulosa cell cultures from different animals in the presence of an individual concentration of FSH from 0-100ng/ml.

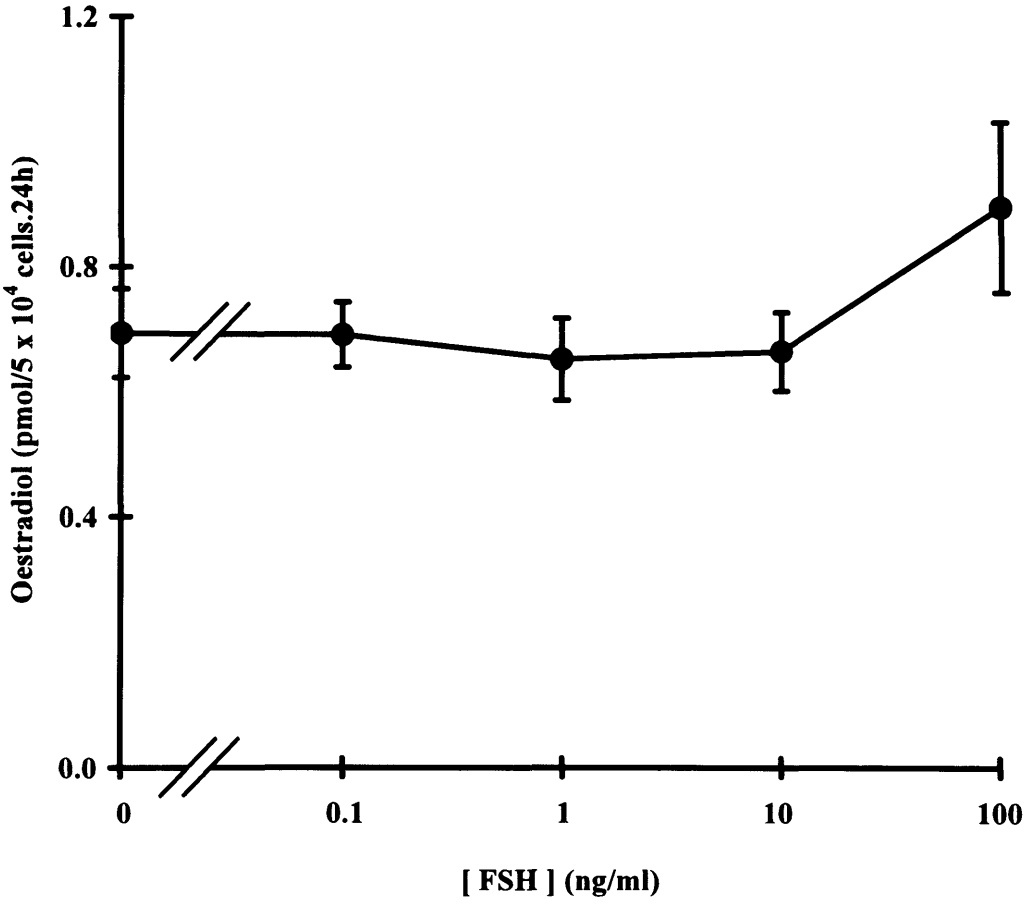


Figure 3.6. Effects of FSH on oestradiol production in porcine granulosa cells isolated from large antral follicles. Each measurement represents the mean (±SEM) oestradiol concentration (pmol/5 x 10^4 viable cells.24h) for 3 independent granulosa cell cultures from different animals in the presence of an individual concentration of FSH from 0 to 100ng/ml.

3.3 Discussion

In this chapter, the expression and activities of the 11βHSD enzymes were investigated in porcine granulosa cells from antral follicles and ovarian cysts. The determination of enzyme activities involved measuring net cortisol oxidation in cultured granulosa cells, as well as testing the cofactor preference of enzymes in granulosa cell homogenates from each follicle category. The effects of FSH on net 11βHSD activities in granulosa cells were also investigated.

Expression studies indicated that transcripts of mRNA encoding 11βHSD1 and 11βHSD2 appeared to be present in porcine granulosa cells from antral follicles, irrespective of follicle size. This pattern of 11βHSD isoform expression in porcine granulosa cells shows similarity with enzyme expression in bovine follicular granulosa cells, where 11βHSD1 and 11βHSD2 are co-expressed (Tetsuka *et al.*, 2003; Thurston *et al.*, 2007), but differs from the exclusive 11βHSD2 expression observed in rat and human follicular granulosa cells. To fully elucidate whether 11βHSD expression in granulosa cells across the porcine ovarian cycle shows a predominant, but not absolute change in enzyme expression after ovulation, it would be necessary to conduct PCR assays with cDNA originating from porcine CL. It would also be interesting to employ quantitative real-time PCR to determine the relative levels of expression of each 11βHSD isoform in porcine granulosa cells from each size of antral follicle.

The absence of detectable net 11-KSR activities in granulosa cells cultured with cortisone, and the lack of NADPH-dependent enzyme activity, irrespective of whether G-6-P was added to granulosa cell homogenates, indicated that the 11βHSD enzymes could act primarily (if not exclusively) as dehydrogenases in porcine granulosa cells. It has been noted, however, that the 11-KSR activity of 11βHSD1 as isolated from rat liver is unstable, particularly in microsomal preparations (Lakshmi and Monder, 1985; Lakshmi and Monder, 1988). Microsomes are vesicles that can form spontaneously from fragments of SER, the intracellular location of 11βHSD1 (Odermatt *et al.*, 1999), during the process of tissue homogenisation. When liver microsomes were exposed to elevated

temperatures and treated with detergents and phospholipases, the reductase activities of 11βHSD1 were lost, however 11β-DH activities were not affected (Lakshmi and Monder, 1985; Lakshmi and Monder, 1988). In a similar way, the application of hypotonic lysis buffer to granulosa cells in the studies reported in the current chapter may have diminished 11-KSR activities in granulosa cell homogenates by disrupting SER membranes. However, in the absence of detergent- or phospholipase-treatment of cells in the present study, the spontaneous formation of microsomes from the SER membrane is likely to have occurred. Thus the microenvironment for 11βHSD1 in the SER should have been restored in the granulosa cell homogenates. In this case, the intralumenal location of the active site of 11βHSD1 may have restricted access of the exogenous NADPH to the enzyme, such that this cosubstrate could not support the reductase activities of 11βHSD1. Nevertheless, reductase activities were still not detected in homogenates incubated with excess exogenous G-6-P. Unlike NADPH, G-6-P would be expected to traverse the microsomal membrane to act as a potential cofactor for H6PDH and stimulate H6PDH activities. H6PDH would thus regenerate NADPH in the SER lumen, where that NADPH should be available to the cofactor binding site of 11βHSD1 to drive 11-KSR activity.

These data support findings discussed in section 1.4.2.2, wherein the 11β-DH activity of 11βHSD1 seems to dominate in steroidogenic gonadal cells from a variety of species (Gao *et al.*, 1997; Michael *et al.*, 1997; Ge and Hardy, 2000; Yong *et al.*, 2000; Tetsuka *et al.*, 2003; Thurston *et al.*, 2007). This may be due to the preferential use of NADPH by the cytochrome P450 (CYP) enzymes in these cells, in turn, producing $NADP^+$ to drive the 11β-DH activity of 11βHSD1 (Michael *et al.*, 2003; Ge *et al.*, 2005). In the current chapter, only the oxidation of cortisol (catalysed by the 11β-DH activity of 11βHSD enzymes) was observed in cultured granulosa cells. In these intact granulosa cells, the steroidogenic activity of functional CYP enzymes would be expected to regenerate $NADP^+$, which can preferentially support the 11β-DH activity of 11βHSD1. Steroid synthesis by CYP enzymes in the granulosa cells may therefore limit NADPH availability within the SER lumen, which could account for the lack of detectable 11-KSR activity of 11βHSD1 displayed in intact granulosa cells in primary

culture. In granulosa cell homogenates however, the actions of the steroidogenic enzymes may have been compromised, thus potentially removing the effect of the $NADP^+$/NAPDH balance that could favour the 11β-DH activities of 11βHSD1 in the intact, functional granulosa cells. Hence the granulosa cell homogenates would have been expected to display 11β-DH or 11-KSR activities of 11βHSD1 in the presence of excess concentrations of either exogenous $NADP^+$ or NADPH, respectively. The addition of exogenous $NADP^+$ was shown to support the net 11β-DH activities of 11βHSD1 in granulosa cell homogenates. On the other hand, the addition of excess exogenous NADPH, even in the presence of G-6-P, could not stimulate the 11-KSR activities of 11βHSD1 in these cell homogenates. The lack of detectable cortisol regeneration in both cultured granulosa cells and granulosa cell homogenates could thus indicate a preference for the ovarian 11βHSD1 enzyme to oxidise cortisol in porcine granulosa cells, which is not simply dependent on cofactor availability.

Net cortisol oxidation increased in granulosa cells with increasing antral follicle size but low levels of cortisol oxidation by 11βHSD were observed in cells from ovarian cysts. This indicates that within small antral follicles and ovarian cysts, mural granulosa cells may experience the highest intracellular concentrations of active glucocorticoid. Small antral follicles and ovarian cysts share a similar property, in that both structures have the greatest potential for follicular expansion and growth. In order to become a preovulatory follicle, each small antral follicle must increase its antral volume by approximately 60-fold, a size-increase comparable to the formation of a spontaneous ovarian cyst from a large antral follicle. Hence, low rates of glucocorticoid oxidation in small antral follicles and ovarian cysts may be functionally linked to follicle or cyst development and/or fluid accumulation in the follicle or cyst antrum.

The progressive increase in net cortisol oxidation with antral follicle growth indicates that intracellular glucocorticoids may be favourable for the development of small antral follicles but less so for large follicles. Glucocorticoids have been shown to stimulate granulosa cell differentiation (Schoonmaker and Erickson,

1983). Therefore, the low levels of cortisol inactivation by 11βHSD that were observed in granulosa cells from small follicles may maintain higher intracellular glucocorticoid concentrations and thus play a role in the differentiation of granulosa cell types in the antral follicle wall during early folliculogenesis. Glucocorticoids have also been reported to inhibit apoptosis in granulosa cells (Sasson *et al.*, 2001). Since follicular atresia appears to occur via granulosa cell apoptosis (Hughes and Gorospe, 1991; Tilly *et al.*, 1992), higher intracellular glucocorticoid concentrations may also be important in limiting atresia in small antral follicles and may also prevent apoptotic degeneration of ovarian cysts.

Glucocorticoids may also regulate follicle development via effects on cell-cell communication, since the expression of the connexin proteins was shown to be increased by the glucocorticoid hormones. Cx26 and Cx32 were shown to be upregulated by dexamethasone in cultured rat hepatocytes (Kwiatkowski *et al.*, 1994). Cx43 expression in human granulosa cells was also increased by dexamethasone, a finding reported by Sasson and Amsterdam (2002) who suggested that the positive effects of dexamethasone on Cx43 expression in gap junctions could be a mechanism by which the glucocorticoids may prevent apoptosis in granulosa cells. Cx26, Cx32 and Cx43 all appear to comprise the gap junctions between granulosa cells in ovarian follicles (see review by Kidder and Mhwai (2002)). The low levels of cortisol oxidation observed in the granulosa cells may therefore play a role in the development of gap junction communications networks between the granulosa cells important for early follicle development and the growth of immature follicles, and could additionally maintain the presence of ovarian cysts.

In porcine large antral follicles, high levels of net cortisol oxidation were observed in the granulosa cells such that low intracellular glucocorticoid levels may be present in preovulatory follicle cells. Cortisol has been shown to inhibit prostaglandin synthesis (Goppelt-Struebe, 1997), which could interfere with the inflammatory event of ovulation (Espey, 1980). Recently Richards (2005) has discussed evidence of a potential role for the prostaglandins, which are

inflammatory mediators, in the process of cumulus expansion. The low intracellular cortisol concentrations in large antral follicles may therefore alleviate any suppressive effects that cortisol may have on prostaglandin production in cumulus expansion and thus ovulation. Low intracellular glucocorticoids in the granulosa cells of large porcine follicles may also be associated with findings that glucocorticoids could inhibit porcine oocyte maturation (Yang *et al.*, 1999). Therefore, the increased inactivation of cortisol by 11βHSD in mural granulosa cells from large follicles may limit exposure of the preovulatory oocyte to glucocorticoids during oocyte maturation. (This point is returned to in chapter 6).

Alongside the determination of levels of net 11β-DH activities in granulosa cells from antral follicles and ovarian cysts, the cofactor preference of the 11βHSD enzymes in porcine granulosa cells was also investigated. Both $NADP^+$- and NAD^+- dependent 11βHSD activities were evident in granulosa cell homogenates, suggesting that both 11βHSD1 and 11βHSD2 activities are present in granulosa cells from ovarian follicles and cysts. Though $NADP^+$ is selective for 11βHSD1 activity and 11βHSD2 activity is NAD^+-dependent, because NAD^+ is a smaller cofactor it may bind to 11βHSD1 or 11βHSD2, as alluded to in section 1.4.2.2. Hence, the inactivation of cortisol in porcine granulosa cell homogenates as a result of NAD^+ addition may reflect steroid metabolism by either 11βHSD isoform while supplemented $NADP^+$ should only increase 11βHSD1 activity.

$NADP^+$- and NAD^+-dependent enzyme activities in porcine granulosa cell homogenates rose 3- and 2-fold, respectively, with increasing follicle diameter, and this was matched by a 3-fold increase in the levels of net cortisol oxidation by cultured granulosa cells with antral follicle growth. This indicated that the 11β-dehydrogenase activities of the 11βHSD enzymes increase in granulosa cells as antral follicles develop and approach ovulation. The low levels of net cortisol oxidation and low NAD^+-dependent 11βHSD activities in granulosa cells from ovarian cysts were comparable to levels observed in granulosa cells from small antral follicles. The comparable activities in these structures may be linked to the

fact that small antral follicles and ovarian cysts share the highest potential for growth, as described above.

While NAD^+-dependent activities in granulosa cells from ovarian cysts were low, $NADP^+$-dependent activities in cells from cysts were as high as enzyme activities exhibited in large antral follicles. This may indicate that the dehydrogenase activity of 11βHSD1 dominates over that of 11βHSD2 in the granulosa cells from large antral follicles and ovarian cysts. Recent studies in rat Leydig cells, however, have identified a possible third isoform of 11βHSD (11βHSD3), which is a high-affinity 11β-dehydrogenase enzyme that, like 11βHSD1, utilises $NADP^+$ (Ge *et al.*, 1997; Ge and Hardy, 2000). The expression of this novel enzyme has not been investigated in the steroidogenic cells of the ovarian follicles therefore, it would be useful to determine whether a proportion of $NADP^+$-dependent activities could be ascribed to 11βHSD3 activity in the granulosa cells. Nevertheless, if the increase in $NADP^+$-dependent activities in granulosa cells with follicle growth could, in part, be attributed to a rise in 11βHSD1 activity then this may also reflect an increase of 11βHSD1 expression as the follicle approaches ovulation. This would appear to correlate with reports that gonadotrophin-treatment was shown to upregulate 11βHSD1 expression in human and granulosa cells (Tetsuka *et al.*, 1997; Tetsuka *et al.*, 1999b). Furthermore, increased 11βHSD1 expression has been reported after ovulation in the granulosa cells of human, rat and bovine ovaries (Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Ricketts *et al.*, 1998; Tetsuka *et al.*, 1999a; Tetsuka *et al.*, 1999b; Tetsuka *et al.*, 2003; Thurston *et al.*, 2007).

As a part of the granulosa cell culture studies conducted in this thesis, cell viability was assessed prior to seeding but not at the end of the 24-hour culture period. Three different dyes that can be used to visually assess cell viability are trypan blue, crystal violet and neutral red. In the final study conducted in chapter 3 of this thesis (described in section 3.2.5), oestradiol production by the cultured granulosa cells did not appear to be stimulated by incubation with increasing concentrations of FSH. In order to rule out the possibility that the cells were undergoing apoptosis, the cells could have been stained with a cell viability dye at

the end of the 24-hour culture period and the percentage viability compared with that measured before the cells were seeded into their culture wells.

As mentioned in section 3.3, porcine granulosa cells were cultured in serum-free medium, which was suggested to uphold oestradiol production by the cells *in vitro* (Picton *et al.*, 1999). The results presented in the paper by Picton *et al.* (1999) show that in the absence of serum, after 144 hours in culture and in the presence of 1μg/l FSH, granulosa cells from small antral follicles produced approximately 23ng/ml oestradiol and granulosa cells from large antral follicles produced around 51ng/ml oestradiol. As described in section 3.2.5 of this thesis, porcine granulosa cells were incubated with 0, 1, 10 and 100ng/ml FSH for 24 hours. Figure 3.6 showed that oestradiol production by these granulosa cells at the higher concentrations of FSH however, did not significantly vary from basal levels of hormone production, that is, those levels occurring in the absence of FSH (0.7pmol/ml oestradiol). The culture medium used by Picton *et al.* (1999) was Dulbecco's modified Eagles medium:Ham's F_{12} medium whereas the culture medium used for the granulosa cell studies presented in this thesis was McCoy's 5A. Though the same supplements were added to the medium, it is possible that the use of McCoy's 5A medium as the base culture medium may not be ideal for the maintenance of oestradiol production by porcine granulosa cells cultured *in vitro*. In addition, a culture period of 24 hours, in this case, may not have been sufficient to observe a significant increase in oestradiol synthesis.

One potential explanation for the lack of FSH-stimulated oestradiol production by these porcine granulosa cells was that the FSH was not sufficiently solubilised in the McCoy's 5A culture medium. Though FSH is soluble in aqueous media, if the glycoprotein hormone was not fully dissolved in solution then this could decrease the amount of FSH to which the granulosa cell-surface FSH receptors could be exposed. Thus hormone-receptor binding may not have been reached its maximum potential to increase oestradiol production. The potential effect of low FSH solubility could have been ascertained by testing dilutions of the culture medium in a bioassay for effects on aromatase activities, or the production of

cAMP or inhibin, as well as that of oestradiol in other steroidogenic cells or tissues, for example, Sertoli cells.

Another possible interpretation of the lack of effects of FSH is that, as granulosa cells were obtained from large porcine antral follicles, these cells may have begun to luteinise in the follicle before they were extracted for culture. This was shown to occur in bovine granulosa cell cultures, where cells became LH- rather then FSH-responsive (O'Shaughnessy *et al.*, 1996; Rajapaksha *et al.*, 1996). In addition, the granulosa cells may have luteinised during the culture period, though McCoy's 5A medium is not that typically used for granulosa-lutein cell culture such as, modified Eagles medium. Furthermore, a longer culture period of 48 hours is usually required for granulosa cell luteinisation to occur *in vitro*. If the granulosa cells in this study were suspected to have undergone luteinisation in culture, then these cells could have been incubated with increasing concentrations of LH, and the stimulation of steroidogenic output by LH could be compared with that resulting from FSH addition. Moreover, the progesterone concentrations in the culture medium of these cells could have been assessed by RIA after 24 hours in culture. These progesterone concentrations could then be compared with the progesterone concentrations in the medium of granulosa cells from small porcine antral follicles cultured for 24 hours. Granulosa cells from immature antral follicles might be expected to have a decreased potential to undergo luteinisation, and could therefore be more likely to maintain a functional follicular phenotype throughout culture.

In summary, the data presented in this chapter indicated that 11βHSD1 and 11βHSD2 were co-expressed in granulosa cells from porcine antral follicles, with both enzymes acting exclusively as 11β-dehydrogenases to inactivate cortisol. The lowest net 11β-dehydrogenase activities were measured in granulosa cells from small antral follicles and ovarian cysts, structures which both had the greatest potential for antral expansion. Antral follicle growth was associated with increasing $NADP^+$- and NAD^+-dependent enzyme activities, and an overall

increase in net 11β-DH activities in granulosa cells, indicating that the highest net cortisol oxidation was demonstrated in preovulatory follicles.

Chapter 4

Effects of Intrafollicular Enzyme Modulators in Antral Follicles of Increasing Size on 11βHSD1 Activities in Rat Kidney Homogenates

4.1 Background

Human, bovine and porcine FF, and the fluid from bovine and porcine spontaneous ovarian cysts, have each been reported to contain compounds that can alter NADP(H)-dependent 11βHSD activities in rat kidney homogenates (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). The intrafollicular enzyme modulators in large antral follicles and ovarian cysts could significantly affect the 11β-dehydrogenase activities of 11βHSD1, but not 11βHSD2, as described in section 1.4.7. Therefore these endogenous compounds appeared to be selective for 11βHSD1. Using reverse phase C18 column chromatography, intrafollicular compounds were eluted from FF or cyst fluid and separated on the basis of their hydrophobicity. The hydrophilic components of the ovarian fluids (eluted at 0-30% (v/v) methanol) could significantly stimulate the NADP(H)-dependent activities of 11βHSD whereas hydrophobic compounds (eluted at 75-90% (v/v) methanol) inhibited enzyme activities. The fluid from porcine and bovine ovarian cysts appeared to contain higher levels of 11βHSD1 inhibitors than were observed in FF from large antral follicles, though the levels of inhibitors were not assessed in the fluid from immature antral follicles (Thurston *et al.*, 2003b).

In these prior studies, radiolabelled steroids were spiked into FF and loaded onto C18 columns to ascertain whether any hormones eluted in the range of the hydrophobic 11βHSD1 inhibitors (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). Radiolabelled cortisol, cortisone, oestradiol and testosterone were shown to elute between 45% and 60% (v/v) methanol. The C21 steroids, progesterone and pregnenolone however, both eluted near the range of fractions of FF that contained the intrafollicular inhibitors of 11βHSD1. Progesterone has been shown to suppress the activities of both 11βHSD1 and 11βHSD2, as described in section 1.4.7. Progesterone was therefore unlikely to be a candidate for the intrafollicular enzyme inhibitors, which were unable to inhibit 11βHSD2 activity. However, a role for progesterone as an ovarian inhibitor of 11βHSD1 could not be entirely ruled out (Thurston *et al.*, 2003b).

The aims of this chapter were therefore:

1. To compare net effects of fluid from small, medium and large antral follicles, and spontaneous ovarian cysts, on the $NADP^+$-dependent oxidation of cortisol by 11βHSD1 in rat kidney homogenates.

2. To compare the effects of the intrafollicular 11βHSD1 modulators, resolved from small, medium and large antral follicles and from ovarian cysts, on $NADP^+$-dependent 11βHSD1 activity in rat kidney homogenates.

3. To assess any correlation between the intrafollicular concentrations of progesterone in antral follicles and ovarian cysts, and the net effects of each fluid on 11βHSD1 activity in rat kidney homogenates.

4.2 Results

4.2.1 Confirmation of the selection of healthy porcine antral follicles

The selection of healthy antral follicles began with a visual assessment according to the criteria outlined by Maxson *et al.* (1985) and Guthrie *et al.* (1995), as described in section 2.2, before each ovarian fluid was aspirated from follicles (detailed in section 2.3). Androstenedione, oestradiol and progesterone concentrations in FF were determined by ELISA or RIA to confirm the morphological assessment of antral follicles deemed to be non-atretic. The ELISA or RIA methods used to measure intrafollicular gonadal steroid concentrations are described in section 2.8.

In antral follicles, androstenedione and oestradiol concentrations increased with follicle diameter ($P<0.05$), though this trend was not observed for progesterone (Table 4.1). Mean intrafollicular concentrations of oestradiol in antral follicles ranged from 64 to 1523nM, which were greater than oestradiol concentrations reported by Maxson *et al.* (1985) in atretic porcine follicles (18±5nM). Likewise, all mean intrafollicular progesterone concentrations measured in samples of FF (754-1338nM) fell within the range previously published for healthy, preovulatory porcine follicles by Conley *et al.* (1994) (219nM to 1945nM). Hence, all fluid samples were confirmed to be aspirated from non-atretic follicles.

4.2.2 Effects of porcine ovarian fluids on $NADP^+$-dependent 11βHSD1 activities in rat kidney homogenates

After confirmation of the health status of antral follicles, each ovarian fluid was incubated (at 10% (v/v) of the final volume) with rat kidney homogenate for 1 hour, following the protocol in section 2.6.2. The resulting 11βHSD1 activities were measured using a radiometric conversion assay (detailed in section 2.5.2) and compared with a control enzyme activity in kidney homogenates measured in the absence of FF/cyst fluid.

All porcine ovarian fluids, irrespective of source, significantly inhibited $NADP^+$-dependent cortisol oxidation over 1 hour in renal homogenates, as compared to control enzyme activities ($P<0.05$; Figure 4.1). The extent of inhibition of 11βHSD1 activity by FF aspirated from antral follicles significantly decreased with increasing antral follicle size ($P<0.01$). Hence, FF from small antral follicles exerted the greatest inhibition of enzyme activities (50±5% inhibition; $P<0.01$), which decreased progressively to only 23±3% inhibition (compared to control enzyme activities) by FF from large antral follicles ($P<0.05$). Fluid aspirated from spontaneous ovarian cysts suppressed the dehydrogenase activity of 11βHSDl by 59±3% of control activity ($P<0.01$). The extent of inhibition exerted by cyst fluid was significantly higher than that exerted by FF from large antral follicles ($P<0.001$), but similar to the inhibitory effects of FF from small antral follicles ($P>0.05$).

4.2.3 Effects of resolved fractions of porcine ovarian fluids on $NADP^+$-dependent 11βHSD1 activities in rat kidney homogenates

Each sample of FF and cyst fluid was subjected to reverse phase column chromatography, whereby fractions of each fluid were eluted by the application of increasing concentrations of 0-100% (v/v) methanol to C18 chromatography columns, as described in section 2.4. As a result, the most hydrophilic compounds were eluted from ovarian fluids at the lowest concentrations of methanol and increasingly hydrophobic components of fluid were eluted at increasing concentrations of methanol.

Fractions eluted at 0% and 10% (v/v) methanol were evaporated to dryness under nitrogen gas, and reconstituted in 0% (v/v) methanol whereas fractions eluted at 20-100% (v/v) methanol were dried down and reconstituted in 20% (v/v) methanol. Each eluted fraction of fluid was tested for effects on $NADP^+$-dependent cortisol oxidation at a final dilution of 10% (v/v). Therefore, the final methanol concentration was ≤2% (v/v) in any sample tube. Control enzyme activities were measured in the absence of FF, but in the presence of 0% (v/v) methanol (for comparison of enzyme activities with fractions of fluid eluted at 0% and 10% (v/v) methanol), or in the presence of 2% (v/v) methanol (for comparison with effects of fractions eluted at 20-100% (v/v) methanol).

The majority of eluted fractions of FF from small antral follicles significantly inhibited the $NADP^+$-dependent oxidation of cortisol (Figure 4.2). To expand on this, fractions of FF eluted between 30% and 85%, and at 95% (v/v) methanol each suppressed $NADP^+$-dependent 11βHSD1 activities by up to 50±4%, compared to control enzyme activity ($P<0.05$). None of the resolved fractions of fluid from small antral follicles stimulated 11βHSD1 activities in rat kidney homogenates.

Fractions of FF eluted from medium antral follicles at 10-100% (v/v) methanol all significantly inhibited $NADP^+$-dependent 11βHSD1 activities by up to 47±6%, relative to control enzyme activity ($P<0.05$; Figure 4.3). As observed for small antral follicles, no fractions of FF eluted from medium antral follicles stimulated cortisol oxidation by 11βHSD1.

Fractions of fluid from large antral follicles eluted between 40% and 100% (v/v) methanol significantly inhibited the $NADP^+$-dependent activity of 11βHSD1 by up to 46±3% ($P<0.01$), compared to control enzyme activity (Figure 4.4). A single hydrophilic fraction of FF, eluted at 0% (v/v) methanol, significantly increased 11βHSD1 activity by 26±5% ($P<0.01$).

Fractions of cyst fluid eluted at 50-100% (v/v) methanol significantly inhibited cortisol oxidation by 11βHSD1 in rat kidney homogenates (Figure 4.5). This inhibition was by up to 49±8% relative to control enzyme activity ($P<0.05$). No evidence of stimulation on $NADP^+$-dependent cortisol oxidation was seen with any eluted fractions of fluid from ovarian cysts.

When C18 columns were loaded with PBS in place of FF or cyst fluid, no eluted fractions of PBS altered the $NADP^+$-dependent activities of 11βHSD1 in rat kidney homogenates compared to control enzyme activities ($P>0.05$; Figure 4.6).

4.2.4 Association of intrafollicular progesterone concentrations in porcine ovarian fluids and the extent of inhibition exerted by fluids on $NADP^+$-dependent 11βHSD1 activities in rat kidney homogenates

There was no correlation between the concentration of progesterone in a given sample of FF or cyst fluid and the inhibitory effect of that fluid on $NADP^+$-dependent 11βHSD1 activities in rat kidney homogenates ($R^2=0.019$; $P=0.560$; Figure 4.7).

Table 4.1. Intra-follicular androstenedione (A4), oestradiol (E2) and progesterone (P4) concentrations (nM) in porcine antral follicles and ovarian cysts

	Hormone concentration (nM)		
Follicle category	A4 (nM)	E2 (nM)	P4 (nM)
Small	223±58[a]	64±14[a]	1161±244[a]
Medium	364±74[a]	141±32[a]	754±110[a]
Large	2257±140[b]	1523±321[b]	1338±121[a]
Ovarian Cyst	523±241[a]	162±133[a]	1099±17[a]

The data indicate the mean (±SEM) intra-follicular hormone concentration for 5 individual porcine FF or cyst fluid samples from different animals. Within each column, data showing different superscripts differ significantly ($P<0.05$).

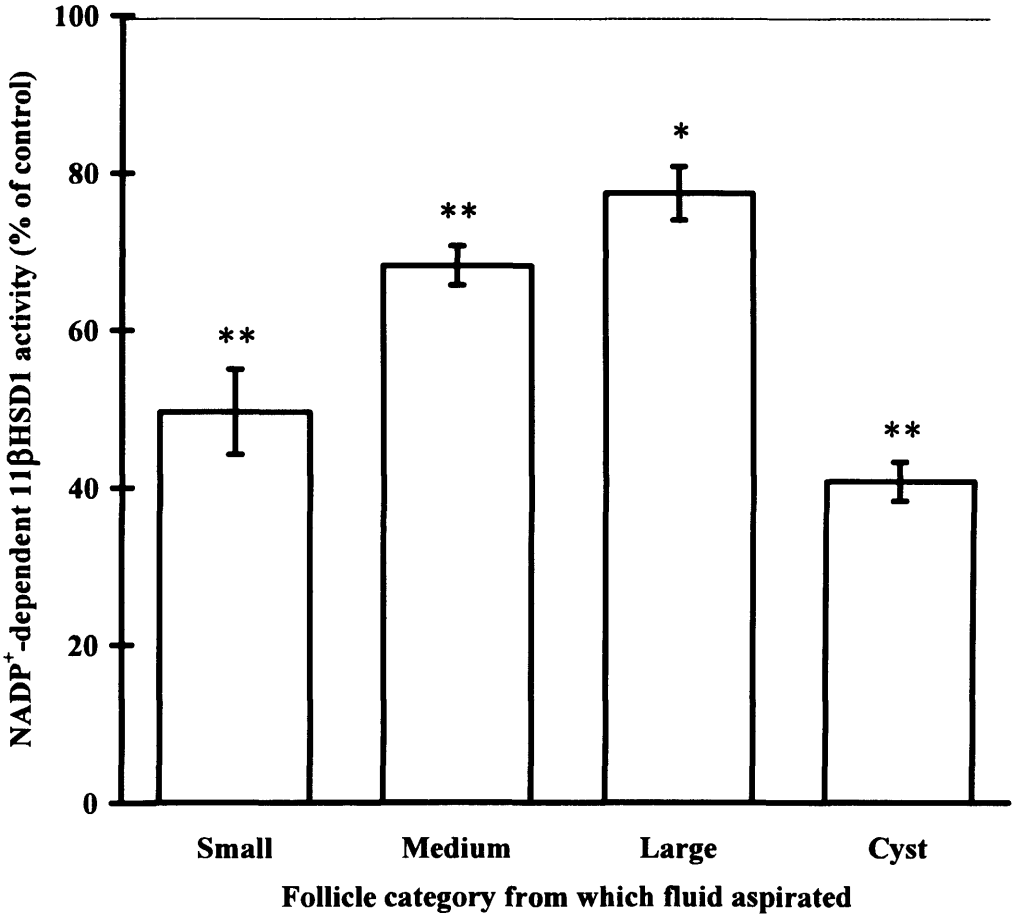


Figure 4.1. Effects of porcine FF from small, medium and large antral follicles and of porcine cyst fluid from spontaneous ovarian cysts on $NADP^+$-dependent cortisol oxidation by 11βHSD1 in rat kidney homogenates. Each data point represents the mean (±SEM) 11βHSD1 activity (% of control) for 5 individual fluid samples from different animals, with a given fluid assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control enzyme activity in rat kidney homogenate of 100%, which equated to 11.2±1.0pmol cortisone/mg tissue.1h. *P<0.05 and **P<0.01 versus respective control enzyme activity measured in the absence of FF or cyst fluid.

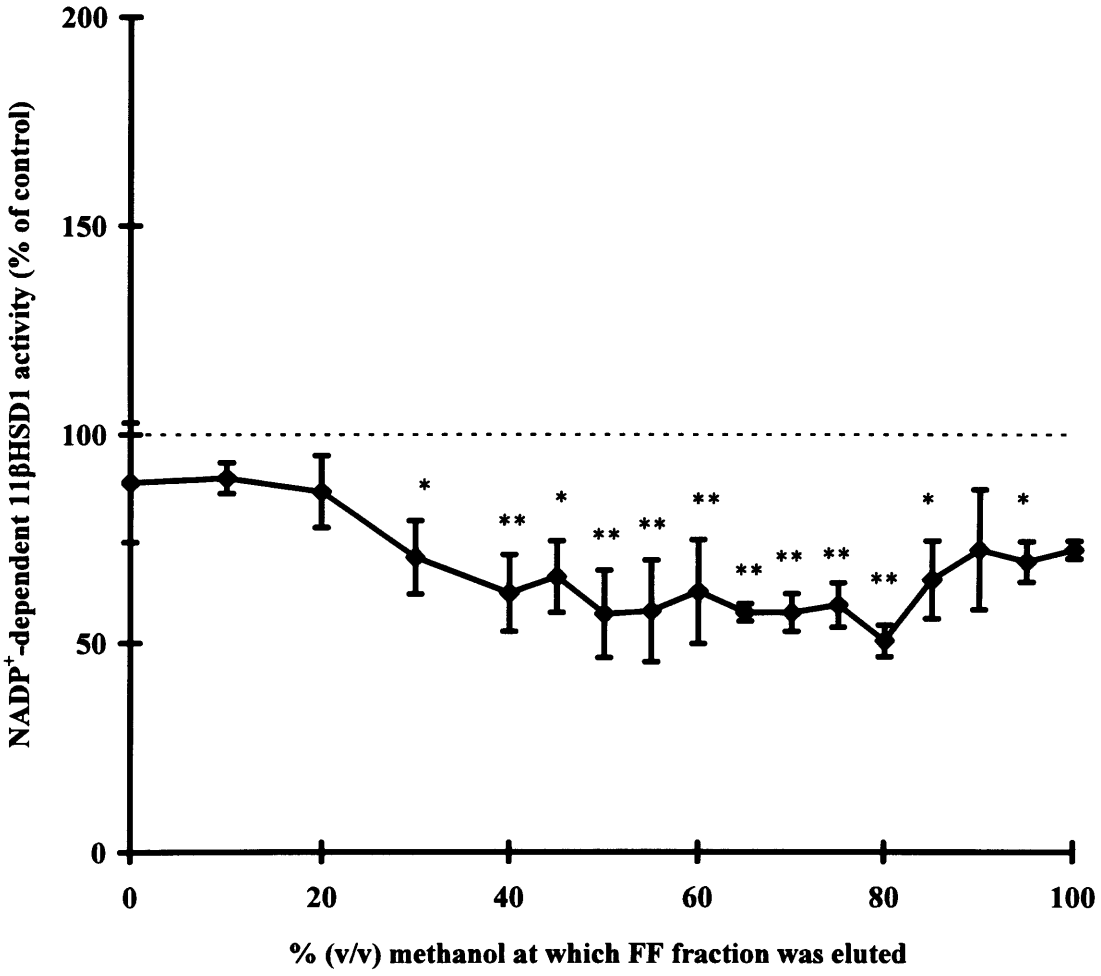


Figure 4.2. Effects of C18 fractions of porcine FF from small antral follicles on NADP$^+$-dependent cortisol oxidation by 11βHSD1 in rat kidney homogenates. Each data point represents the mean (±SEM) 11βHSD1 activity (% of control) for 5 individual FF samples from different animals, with a given FF fraction assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control enzyme activity of 100%, measured in the absence of FF, which equated to 7.0±0.3pmol cortisone/mg tissue.1h. *$P<0.05$ and **$P<0.01$ versus control enzyme activity measured in the absence of FF fractions.

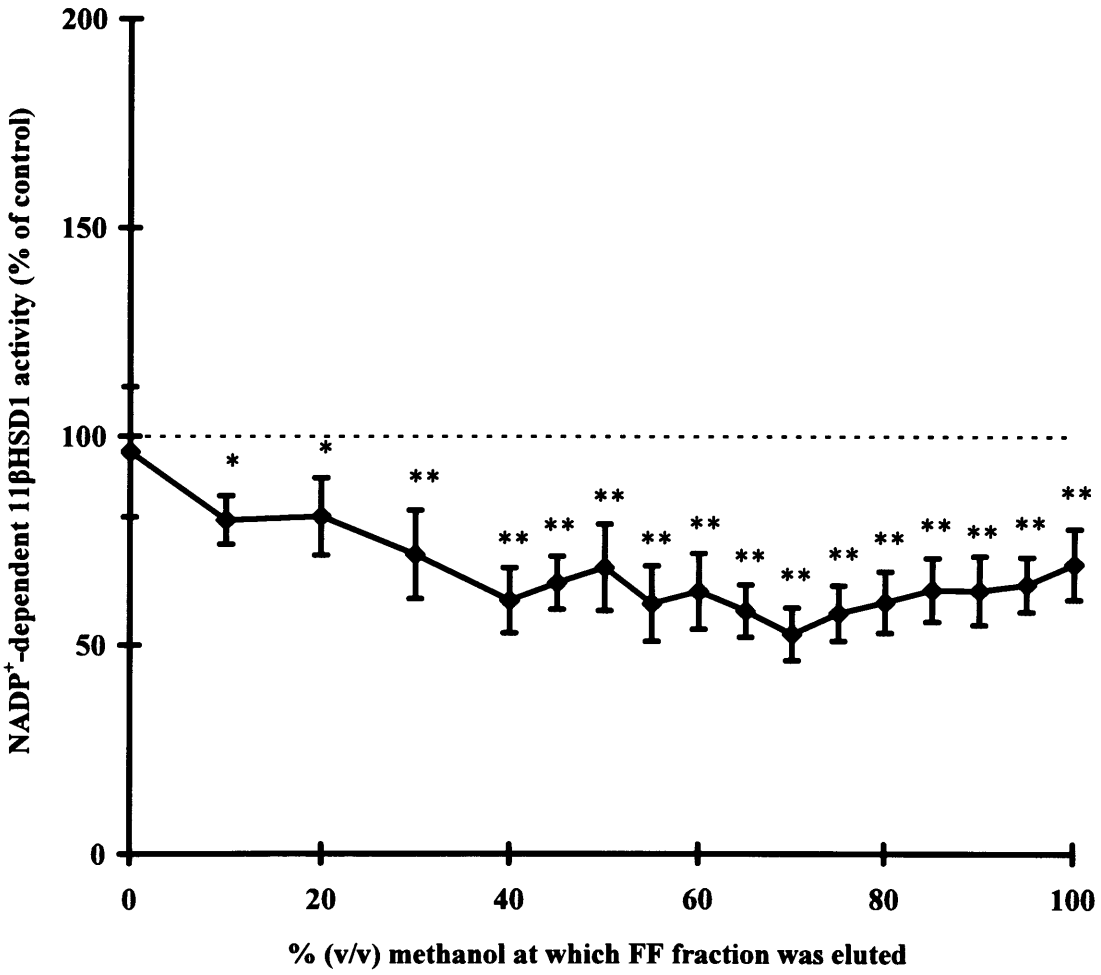


Figure 4.3. Effects of C18 fractions of porcine FF from medium antral follicles on NADP$^+$-dependent cortisol oxidation by 11βHSD1 in rat kidney homogenates. Each data point represents the mean (±SEM) 11βHSD1 activity (% of control) for 5 individual FF samples from different animals, with a given FF fraction assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control enzyme activity of 100%, which equated to 8.5±0.8pmol cortisone/mg tissue.1h. *P<0.05 and **P<0.001 versus control enzyme activity measured in the absence of FF fractions.

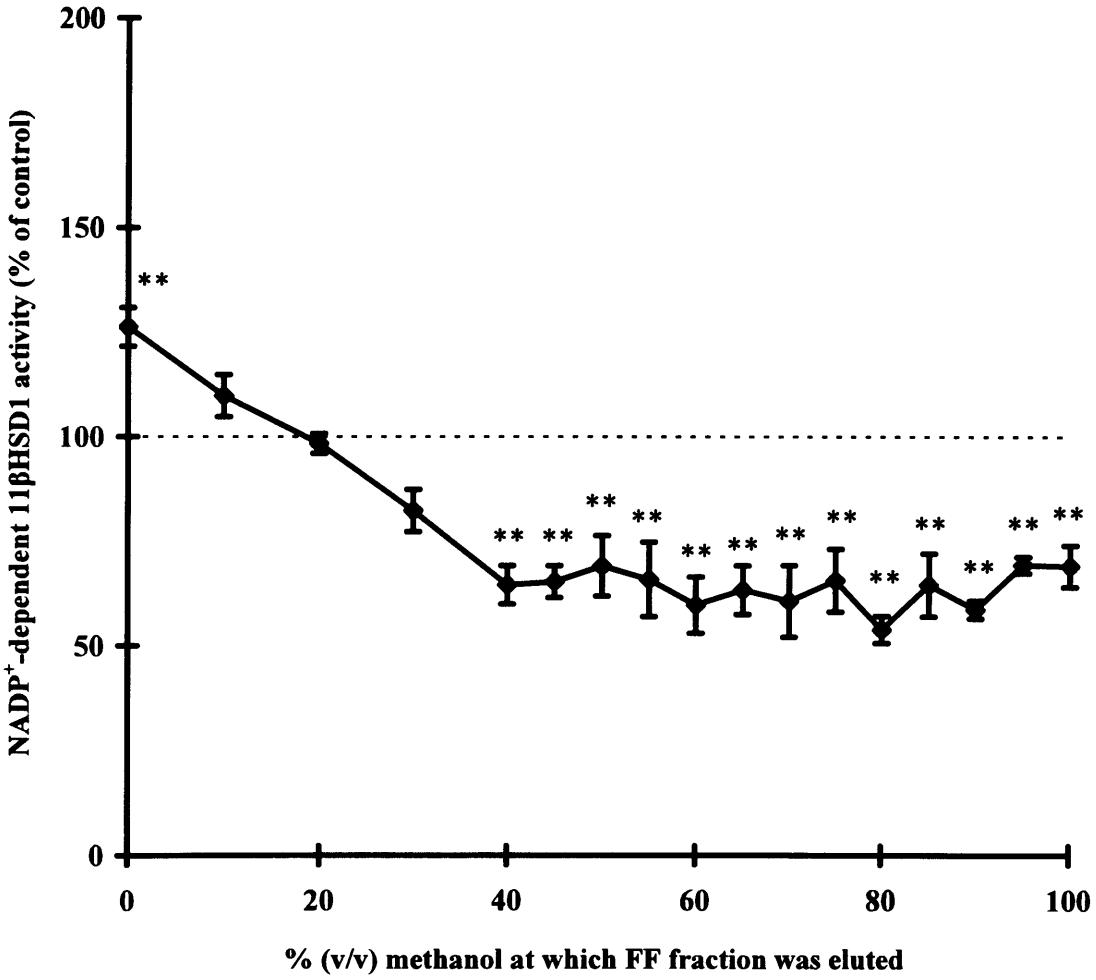


Figure 4.4. Effects of C18 fractions of porcine FF from large antral follicles on NADP$^+$-dependent cortisol oxidation by 11βHSD1 in rat kidney homogenates. Each data point represents the mean (±SEM) 11βHSD1 activity (% of control), calculated for 5 individual FF samples from different animals, with a given FF fraction assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control enzyme activity of 100%, measured in the absence of FF, which equated to 6.2±0.3pmol cortisone/mg tissue.1h. **P<0.01 versus control enzyme activity measured in the absence of FF fractions.

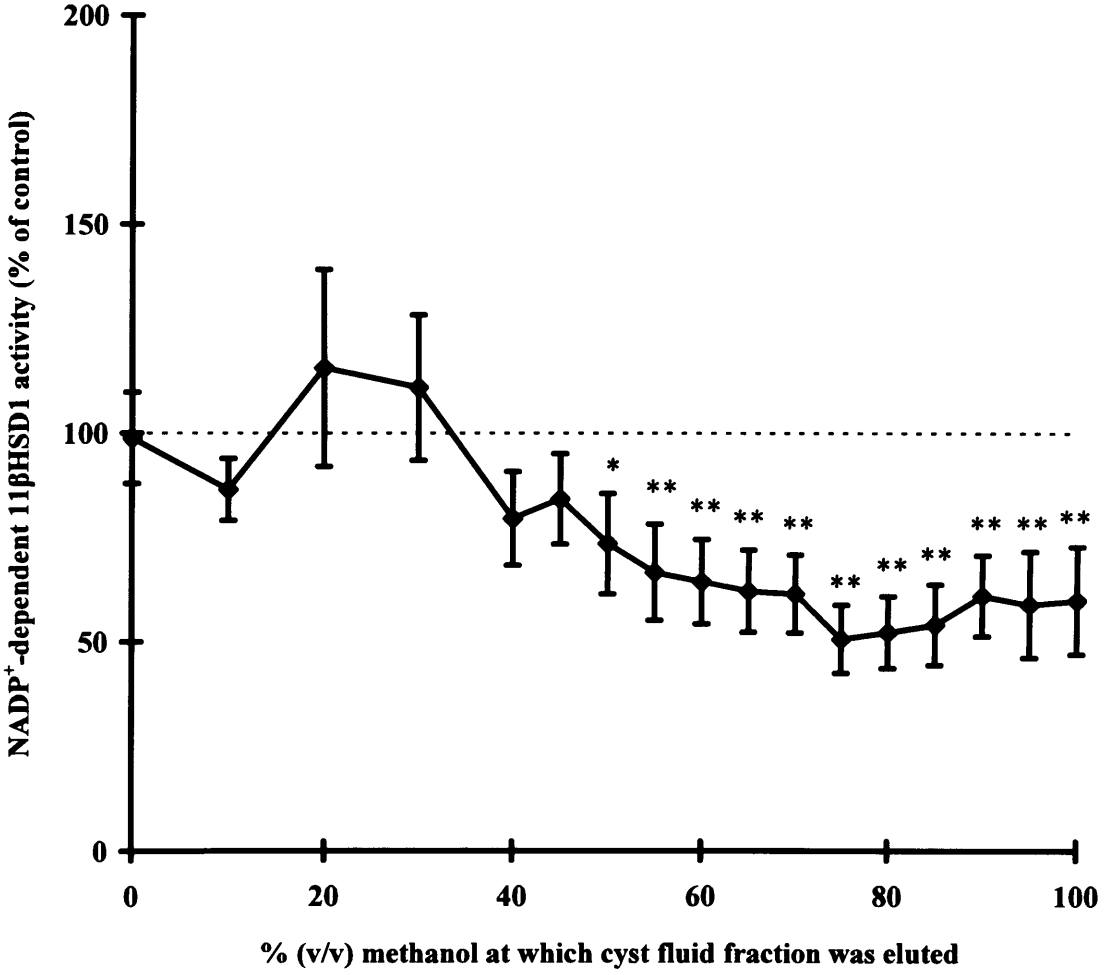


Figure 4.5. Effects of C18 fractions of porcine cyst fluid from spontaneous ovarian cysts on $NADP^+$-dependent cortisol oxidation by 11βHSD1 in rat kidney homogenates. Each data point represents the mean (±SEM) 11βHSD1 activity (% of control) for 5 individual cyst fluid samples from different animals, with a given cyst fluid fraction assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control enzyme activity of 100% which equated to 12.3±1.3pmol cortisone/mg tissue.1h. *$P<0.05$ and **$P<0.01$ versus control enzyme activity measured in the absence of cyst fluid fractions.

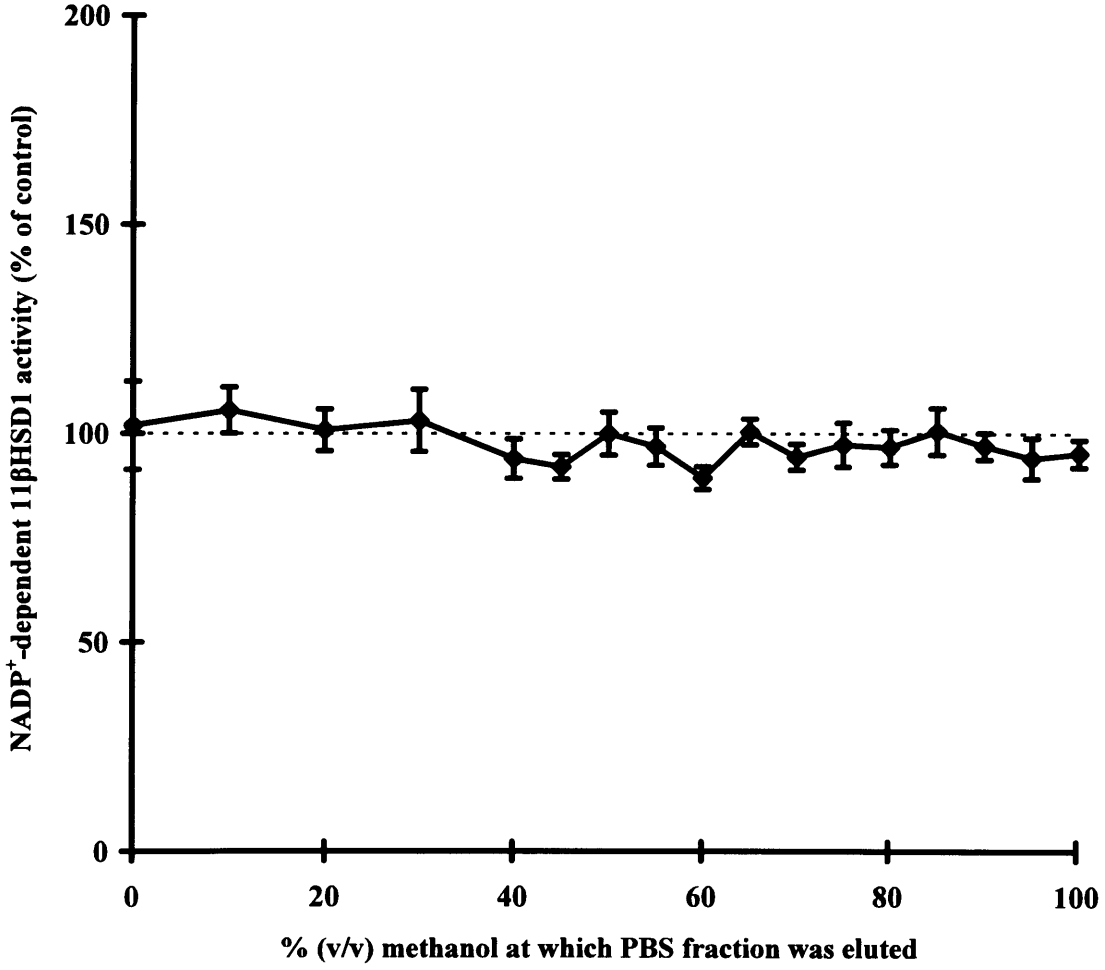


Figure 4.6. Effects of C18 fractions of PBS on $NADP^+$-dependent cortisol oxidation by 11βHSD1 in rat kidney homogenates. Each data point represents the mean (±SEM) 11βHSD1 activity (% of control) for 5 individual PBS samples, with a given sample assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control enzyme activity of 100%, which equated to 11.4±1.4pmol cortisone/mg tissue.1h.

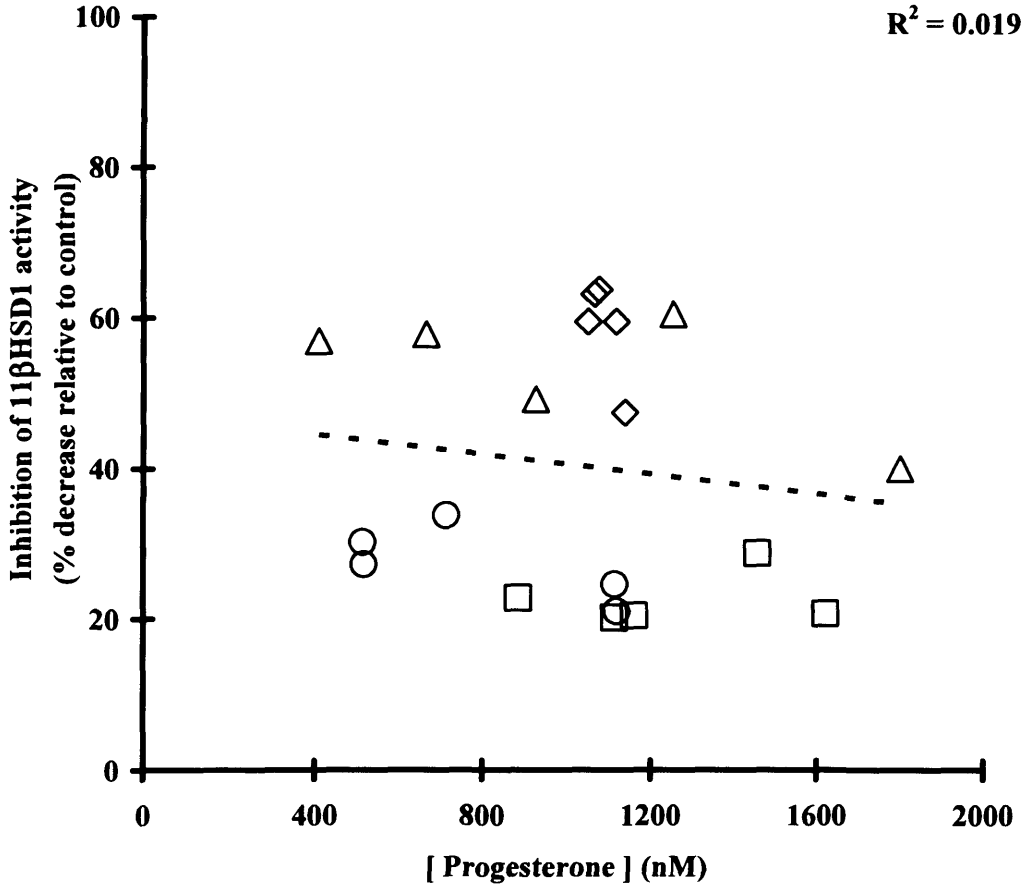


Figure 4.7. Correlation between intrafollicular progesterone concentrations and the percentage inhibition of $NADP^+$-dependent 11βHSD1 activities in rat kidney homogenates by the respective fluids from each follicle category. Progesterone concentrations and the extents of inhibition of 11βHSD1 activity were each measured in the fluids from 5 porcine small (Δ), 5 medium (○) and 5 large (□) antral follicles and from 5 spontaneous ovarian cysts (◇).

4.3 Discussion

In the studies reported in this chapter, FF from healthy antral follicles and fluid from spontaneous ovarian cysts significantly suppressed $NADP^+$-dependent cortisol oxidation by 11βHSD1 in rat kidney homogenates. There was a decrease in the extent of enzyme inhibition exerted by FF from antral follicles with increasing follicle size. Interestingly, cyst fluid exerted a greater inhibitory effect on renal 11βHSD activities than FF from large antral follicles, though cyst fluid had similar inhibitory effects to FF from small follicles. When considering antral follicles alone, the least inhibitory effects were exerted on net cortisol oxidation by FF from large antral follicles, compared to small antral FF. This decrease in enzyme inhibition could have been due to a greater dilution of the intrafollicular enzyme inhibitors within the larger follicles which contain a greater volume of antral fluid. However, given that cyst fluid suppressed 11βHSD1 activities to a greater extent than large antral FF, though the antral volume of an ovarian cyst is approximately 100-fold greater than that of a large antral follicle, a dilution effect would be unlikely. Alternatively, the decreasing inhibitory effects on 11β-DH activity by FF from increasing follicle size may reflect a decrease in the local synthesis of the hydrophobic inhibitors of 11βHSD1 with follicle growth. Thus, the highest levels of intrafollicular 11βHSD1 inhibitors could be present in small antral follicles, with the lowest levels of enzyme inhibitors in large antral follicles.

It is possible that the highly inhibitory effects of fluids from small antral follicles and ovarian cysts on net dehydrogenase activities may be related to the influence of the glucocorticoids on the development each follicular structure, as considered in chapter 3. Both small antral follicles and spontaneous ovarian cysts have a high potential to undergo follicular development and expansion, for which low levels of local cortisol oxidation may be beneficial. As discussed in chapter 3, glucocorticoids can stimulate the differentiation of granulosa cells (Schoonmaker and Erickson, 1983) and inhibit granulosa cell apoptosis (Sasson *et al.*, 2001; Sasson and Amsterdam, 2002). Thus the high inhibitory effects of FF from small antral follicles on cortisol inactivation may serve to limit cortisol metabolism and in so doing, increase intracellular glucocorticoid concentrations in immature

follicles. Consequently, this may promote early antral folliculogenesis through the differentiation of granulosa cells in the follicle wall and the prevention of follicle atresia.

The highly suppressive effects of cyst fluid on net cortisol oxidation by 11βHSD may also increase glucocorticoid concentrations within the mural granulosa cells of ovarian cysts, thereby inhibiting granulosa cell apoptosis in these follicular structures. This could, in turn, limit cyst degeneration and thus play a role in cystic ovarian disease (COD). Furthermore, glucocorticoids have been shown to modify ovarian steroidogenesis (Hsueh and Erickson, 1978; Michael *et al.*, 1993b; Jana *et al.*, 2005). Interestingly, porcine ovarian cysts displayed altered steroid concentrations in homogenates of the follicle wall cells and in the cyst fluid, compared those in antral follicles (Jana *et al.*, 2005). Since the findings reported in this chapter indicated that cyst fluid had high inhibitory effects on 11βHSD activities, the follicular cells in cysts may be exposed to greater glucocorticoid concentrations, potentially affecting ovarian steroidogenesis and contributing to COD.

The decreased extent of inhibition of cortisol oxidation exerted by FF from large antral follicles implies that the low levels of intracellular cortisol might be present in the mural granulosa cells of large antral follicles. This finding could be related to the negative effects of glucocorticoids reported on porcine oocyte maturation (Yang *et al.*, 1999; Chen *et al.*, 2000). The decreased inhibition of 11βHSD activity in the granulosa cells from preovulatory follicles may thus be important for increasing cortisol activation and so limiting the exposure of the oocyte to intrafollicular glucocorticoids in the late stages of oocyte maturation.

In studies conducted in this chapter involving the use of reverse phase C18 column chromatography, the resolved fractions of PBS and double-distilled water had no effects on net cortisol oxidation in renal homogenates, thus any potential effects of the components of the C18 columns in fractions of fluid could be excluded. Each of the fluids isolated from ovarian follicles and cysts, however,

were shown to contain intrafollicular inhibitors of the $NADP^+$-dependent activities of 11βHSD1 in rat kidney homogenates. Prior to fractionation, the extent of enzyme inhibition exerted by porcine ovarian fluids decreased with increasing antral follicle diameter, though a significantly increased extent of inhibition was exerted by the fluid from ovarian cysts. After C18 column fractionation of the porcine ovarian fluids however, the inhibitory fractions eluted from each fluid suppressed enzyme activities in renal homogenates to the similar extents, irrespective of the source of the antral fluid. The elution of the components from FF of large antral follicles did, nevertheless, reveal the presence of relatively hydrophilic enzyme stimulator(s), eluted in a single hydrophilic FF fraction at 0% (v/v) methanol. Therefore, the decreased extent of inhibition of 11βHSD1 activity exerted by FF from large follicles, prior to fractionation, may have resulted of the stimulatory hydrophilic compound(s) counteracting the effects of the hydrophobic inhibitors of 11βHSD1 in this fluid.

The findings of studies conducted in this chapter indicated that the intrafollicular enzyme inhibitors were eluted from porcine FF, irrespective of antral follicle diameter, at 30-100% (v/v) methanol. The findings of previous studies carried out in our laboratory documented that the intrafollicular 11βHSD1 inhibitors eluted from porcine large antral FF at 80-90% (v/v) methanol (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). Therefore for the studies reported herein, the enzyme modulators appeared to elute over a broader range of methanol concentrations than those previously reported by our research group. In the previously published studies, radiolabelled cortisol, cortisone, oestradiol, testosterone and progesterone were found to elute from FF between 40-65% (v/v) methanol which, according to the more recent findings reported in this chapter, were within the range of methanol concentrations (30-100% (v/v) methanol) shown to contain endogenous 11βHSD1 inhibitors. Any of the above steroids might therefore be considered as potential candidates for the intrafollicular inhibitors of 11βHSD1 in porcine ovarian fluids. This would need to be tested by spiking the above hormones into FF and, using C18 columns, eluting each steroid into a different fraction of FF. The steroids could then be individually eluted and incubated with rat kidney

homogenates to assess effects on NADP(H)-dependent activities in the homogenates after 1 hour.

The previous studies conducted in our laboratory also reported that progesterone was eluted from FF at 70% (v/v) methanol, which was just before the range of fractions in which the intrafollicular enzyme inhibitors were eluted from FF and cyst fluid (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). However, progesterone can inhibit both 11βHSD1 and 11βHSD2 activities *in vitro* (Souness *et al.*, 1995; Souness and Morris, 1996; Sun *et al.*, 1998; Quinkler *et al.*, 1999; Thurston *et al.*, 2002). Since the intrafollicular enzyme inhibitors could not inhibit 11βHSD2 activity in renal homogenates, progesterone was not thought to be a likely candidate for the predominant enzyme inhibitor in fluid from human, bovine and porcine antral follicles (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). Data presented in the current chapter showed no correlation between concentrations of progesterone in FF or cyst fluid and the extent of inhibition of 11βHSD1 activities in rat kidney homogenates by the same fluid samples. This finding, like those of Thurston *et al.* (2003b), indicates that progesterone is almost certainly not a candidate for the predominant intrafollicular inhibitor of 11βHSD1.

Although progesterone does not appear to be the main intrafollicular 11βHSD1 inhibitor, the findings of this chapter and those previously reported by our group indicate that the endogenous enzyme inhibitors are hydrophobic compounds (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). Therefore they may be steroids, such as cortisol, cortisone, oestradiol and testosterone as discussed above, or sterols. Additionally, the wide range of methanol concentrations in which endogenous 11βHSD1 inhibitors from porcine ovarian fluids were eluted according to the findings documented in this chapter, has suggested that the enzyme inhibitors in porcine antral follicles might elute as a set of steroid or sterol metabolites with different chemical structures and thus varying degrees of hydrophobicity. Published studies have documented the existence of hydrophobic substrates for renal and hepatic 11βHSD1 other than the glucocorticoids. These additional substrates are all derived from cholesterol and would thus be

hydrophobic. The first examples of possible additional substrates are the dehydroepiandrosterone (DHEA) metabolites, 7α- and 7β-hydroxy-dehydroepiandrosterone (7α- and 7β-OH-DHEA). 11βHSD1 was shown to convert 7α-OH-DHEA to 7-oxo-DHEA in rat, human and pig liver, rat kidney (Robinzon *et al.*, 2003) and in pig kidney (Robinzon and Prough, 2005). The inter-conversion of 7α- and 7β-OH-DHEA with 7-oxo-DHEA was also catalysed by recombinant human 11βHSD1 protein expressed in yeast microsomes (Muller *et al.*, 2006). DHEA has also been shown to decrease the 11-KSR activity of 11βHSD1 in mouse adipocytes (Apostolova *et al.*, 2005) and in rat liver (Gu *et al.*, 2003). In addition, 11βHSD1 appears to be involved in oxysterol metabolism, as the enzyme was shown to reduce 7-ketocholesterol to 7α- and/or 7β-hydroxy-cholesterol in mouse, rat and human liver (Hult *et al.*, 2004; Schweizer *et al.*, 2004). One other group of hydrophobic compounds which have been shown to inhibit 11βHSD1 activity in rat kidney are the bile acids and their amidate derivatives, the mono-, di- and trihydroxylated bile acids (Perschel *et al.*, 1991; Latif *et al.*, 1994).

As these afore-mentioned hydrophobic compounds are hormone metabolites and acid derivatives with different chemical structures, they would be expected to elute across a range of methanol concentrations, as shown in the findings of studies in this chapter. The conversion of 7α-OH-DHEA to 7-oxo-DHEA by 11βHSD1 and the inhibition of 11βHSD1 activity by bile acids and their derivatives were both shown to occur in rat kidney microsomes. In the studies conducted in the current chapter, homogenates of rat kidney were used as the source of $NADP^+$-dependent 11βHSD1 activities. Therefore, if DHEA, oxysterols, bile acids, and/or their metabolites were intrafollicular 11βHSD1 inhibitors, they could be expected to inhibit enzyme activities in rat kidney homogenates as shown in the studies reported in this chapter.

Any one of the established hydrophobic inhibitors of 11βHSD1 discussed above may enter FF or cyst fluid in serum transudate from the circulation. Alternatively the intra-follicular enzyme inhibitors could be synthesised locally by the cells of

the ovarian follicle or cyst. Cholesterol metabolism has been shown to occur in the ovaries of a variety of species. Ovarian mitochondria (isolated from gonadotrophin-treated rats) were able to convert radiolabelled cholesterol into radiolabelled 26-hydroxy-cholesterol in human granulosa cells (Rennert *et al.*, 1990). This conversion of cholesterol to a more hydrophilic oxysterol metabolite is catalysed by CYP26 (26-hydroxylase), the mRNA for which has been identified in human granulosa-lutein cells and in rat ovary (Rennert *et al.*, 1990; Su *et al.*, 1990). Interestingly, CYP26 is also involved in bile acid synthesis (Bjorkhem and Danielsson, 1974), as is CYP7A1 (7α-hydroxylase) (Axelson *et al.*, 1991). CYP7A1 can catalyse the conversion of 7α-hydroxy-cholesterol to 7α-hydroxy-4-cholesten-3-one, a reaction which was shown to occur in pig ovary microsomes (Furster, 1999). Moreover, CYP7B1 (7β-hydroxylase) was shown to catalyse the 7α-hydroxylation of DHEA and 25-hydroxy-cholesterol in the rat prostate (Martin *et al.*, 2001) and CYP7B1 mRNA transcripts were detected in human ovary homogenates (Wu *et al.*, 1999). The cited studies thus indicate that the cells of the ovarian follicles have the capacity to metabolise cholesterol and/or DHEA to a number of hydrophobic metabolites which could serve as intrafollicular hydrophobic inhibitors of the 11βHSD1 enzyme.

As well as there being reports of cholesterol metabolism in ovarian follicles, the presence of hydrophobic compounds that are produced upstream during cholesterol biosynthesis have also been document in ovarian fluids. These intermediate lipid substrates, which are synthesised *en route* from acetate to cholesterol, may also be able to inhibit 11βHSD1 activity in ovarian follicle cells by competing with the glucocorticoids, or other potential physiological substrates, to occupy the active site of the enzyme. One such example is FF-meiosis activating sterol (FF-MAS) (Byskov *et al.*, 1995), which is present in FF and has been shown to induce oocyte maturation and/or improve the developmental potential of oocytes from mice (Grondahl *et al.*, 1998), humans (Grondahl *et al.*, 2000), cows (Donnay *et al.*, 2004) and pigs (Algriany *et al.*, 2004; Faerge *et al.*, 2006). Published work regarding FF-MAS, conversely, shows that the accumulation of this sterol was increased by a rise in LH (Cao *et al.*, 2004) around the time of ovulation (Baltsen, 2001). The results reported in this current chapter

indicated that the highest levels of the intrafollicular 11βHSD1 inhibitors were present in small antral follicles, whereas the lowest levels of enzyme inhibitors were found in preovulatory follicles. Thus, FF-MAS which would be expected to be highest in the FF from large antral follicles, would appear an unlikely candidate for the main intrafollicular 11βHSD1 inhibitor.

The findings in this chapter appear to show an inverse correlation between the suppressive effects of the ovarian fluids on 11β-dehydrogenase activities of 11βHSD1 in rat kidney homogenates, and the levels of net cortisol oxidation in granulosa cells isolated from the corresponding follicular structures (presented in chapter 3). To expand on this, there was a decrease in the extent of inhibition of 11β-DH activities in renal homogenates by FF from antral follicles of increasing size (shown in this chapter), concurrent with increasing levels of net cortisol inactivation by 11βHSD in the mural granulosa cells (described in chapter 3). Moreover, a high level of suppression on the $NADP^+$-dependent activities of 11βHSD1 was exerted by the fluid from ovarian cysts wherein the granulosa cells exhibited low levels of net cortisol oxidation. Thus, the intrafollicular 11βHSD1 inhibitors in the antrum may regulate cortisol metabolism in the granulosa cells if the enzyme inhibitors in the antrum were able to act in a paracrine manner on 11βHSD activities in the follicle wall cells. Hence, this is the subject of the studies reported in the next chapter.

Chapter 5

Effects of Intrafollicular Enzyme Modulators in Porcine Ovarian Fluids on 11βHSD Activities in Porcine Granulosa Cells

5.1 Background

The findings of the study reported by Michael *et al.* (1996) suggested that human granulosa-lutein cells may produce a paracrine factor that could suppress the 11βHSD activities of other granulosa-lutein cells in the same culture. In this study, the net activities of 11βHSD were measured in the granulosa-lutein cells aspirated from single ovarian follicles and in granulosa-lutein cells that were pooled across several follicles and co-cultured. The 11βHSD activities observed in the co-cultured granulosa-lutein cells from different follicles were consistently lower than the expected average enzyme activities calculated from the granulosa-lutein cells of each individual follicle.

The work subsequently published by Thurston *et al.* (2002) showed that FF from human antral follicles wherein the granulosa-lutein cells exhibited low 11βHSD activities could exert a significant inhibitory effect on $NADP^+$-dependent cortisol oxidation in rat kidney homogenates. Conversely, FF aspirated from follicles in which the granulosa cells displayed high 11βHSD activities had no effect on 11βHSD activities in renal homogenates. Porcine , bovine and human FF, as well as bovine and porcine cyst fluid were later shown to contain hydrophobic inhibitors of the NADP(H)-dependent activities of 11βHSD1 in renal homogenates (Thurston *et al.*, 2002; Thurston *et al.*, 2003b).

The results presented in chapter 4 of this thesis indicated a decreasing extent of inhibition of $NADP^+$-dependent 11βHSD activities in rat kidney homogenates by FF from porcine antral follicles of increasing size. Cyst fluid exerted a significantly greater inhibition of $NADP^+$-dependent enzyme activities in renal homogenates than FF from large follicles. Changes in the degree of suppression of enzyme activities by the ovarian fluids appeared to be inversely related to the increasing levels of net cortisol oxidation in granulosa cells with antral follicle growth, and the decreased enzyme activities in granulosa cells from ovarian cysts (shown in chapter 3). This raised the possibility that net cortisol metabolism in the mural granulosa cells may be regulated by the intrafollicular inhibitors of 11β-DH

activities, if enzyme modulators in the antral fluid could act in a paracrine manner on 11βHSD activities in granulosa cells in the follicle or cyst wall.

A number of studies have measured the intrafollicular ratios of cortisol:cortisone as an indication of the *in vivo* metabolism of cortisol in ovarian follicles (Andersen *et al.*, 1999; Michael *et al.*, 1999; Keay *et al.*, 2002; Lewicka *et al.*, 2003; Thurston *et al.*, 2003c). Thus, a further indication of any potential paracrine effects of the inhibitors of 11βHSD1 in FF and cyst fluid on the levels of cortisol oxidation in granulosa cells from ovarian follicles and cysts may be provided by the assessment of the cortisol:cortisone ratios in FF and cyst fluid.

The aims of this chapter were therefore:

1. To assess whether FF or cyst fluid, as well as the resolved fractions of each ovarian fluid, could alter 11βHSD activities in mural granulosa cells from antral follicles and ovarian cysts.

2. To measure the concentrations of cortisol and cortisone, and the intrafollicular ratios of cortisol:cortisone, in the fluids from antral follicles and spontaneous ovarian cysts.

5.2 Results

5.2.1 Effects of porcine ovarian fluids on 11βHSD activities in granulosa cells from porcine antral follicles and ovarian cysts

The effects of porcine FF or cyst fluid on ovarian 11β-DH activities were assessed using porcine granulosa cells from small, medium and large antral follicles and ovarian cysts. The fluids from large antral follicles and ovarian cysts were selected for testing based on the findings that large antral FF and cyst fluid appeared to contain the lowest and highest levels, respectively, of the endogenous ovarian 11βHSD1 inhibitors. In addition, ample quantities of fluid could be obtained from a single large follicle/cyst thus avoiding the need to pool fluids from multiple antral follicles.

Granulosa cells were isolated from each size of antral follicle, and from ovarian cysts, and then seeded into 24-well plates at a density of 5 x 10^4 viable cells/ml of culture medium per well. Cells were cultured for a total of 24 hours, the last 4 hours of which occurred in the presence of medium alone (control) or with FF or cyst fluid, each at a final dilution of 10% (v/v) in the well. 11βHSD activities were measured over the final 4 hours of the culture period using a radiometric conversion assay (detailed in section 2.5.2), and compared with the control enzyme activities in granulosa cells incubated without FF or cyst fluid.

Both FF and cyst fluid significantly suppressed net cortisol oxidation by 11βHSD in porcine granulosa cells from antral follicles and ovarian cysts, compared with enzyme activities measured in the absence of either ovarian fluid (Figure 5.1). FF from large antral follicles exerted a comparable inhibition of between 36% and 46% of enzyme activities in granulosa cells, irrespective of the source of granulosa cells ($P<0.05$ in all cases). In contrast, a progressively increasing extent of inhibition of 45% to 74% was exerted by cyst fluid on net cortisol oxidation in granulosa cells with increasing follicle size ($P<0.01$, irrespective of the follicle origin). Cyst fluid exerted a similar extent of inhibition on 11β-DH activities in granulosa cells from large follicles (by 73±4%) and ovarian cysts (by 74±9%), though the control enzyme activities in granulosa cells from cysts (0.6±0.1pmol cortisone/4h) were around half of those in large follicles (1.4±0.1pmol cortisone/4h).

5.2.2 Effects of resolved fractions of porcine FF from large antral follicles on 11βHSD activities in granulosa cells from porcine large antral follicles

Samples of porcine FF from large antral follicles were subjected to reverse phase C18 column chromatography, as described in section 2.4, to generate resolved fractions of fluid eluted at 0, 20, 40 and 60-100% (v/v) methanol. Each FF fraction was individually incubated with granulosa cells from large follicles, at 10% (v/v) of the final volume in each well, for the last 4 hours of a 24-hour culture period. 11βHSD activities were measured in granulosa cells from large antral follicles as findings in chapter 3 indicated that these granulosa cells

displayed the highest levels of net cortisol oxidation. Furthermore, sufficient numbers of viable granulosa cells could be obtained for primary cultures from one ovarian follicle per animal and the pooling of granulosa cells from multiple follicles was not necessary. Likewise, the effects of large antral FF were tested on enzyme activities because volumes of fluid from a single large antral follicle on one ovary from each animal were sufficient to generate the full range of eluted fractions described above.

As for the assessment of 11βHSD activities in rat kidney homogenates, fractions of FF eluted at 0% (v/v) methanol were evaporated to dryness under nitrogen and reconstituted in 0% (v/v) methanol and fractions eluted at ≥20% (v/v) methanol were reconstituted in 20% (v/v) methanol. Therefore, there was a final concentration of ≤2% (v/v) methanol in all samples. Control enzyme activities in porcine granulosa cells were assessed in the absence of resolved fractions of FF. For comparison with enzyme activities in granulosa cells incubated with fractions of FF eluted at 0% (v/v) methanol, control 11βHSD activities were measured in granulosa cells in the presence of 0% (v/v) methanol. 11βHSD activities measured in granulosa cells incubated with fractions of FF eluted at 20-100% (v/v) methanol were compared with control enzyme activities determined in cells cultured in the presence of 2% (v/v) methanol.

Fractions of FF from large antral follicles eluted at 20% and 60-100% (v/v) methanol significantly inhibited net cortisol oxidation by 11βHSD in granulosa cells by up to 77±8%, compared to control enzyme activity ($P<0.05$; Figure 5.2). Fractions of large antral FF eluted at 0% and 40% methanol appeared to lower 11βHSD activities in granulosa cells compared to control enzyme activity, but this inhibition did not attain statistical significance.

5.2.3 Effects of resolved fractions of porcine cyst fluid on 11βHSD activities in granulosa cells from porcine large antral follicles

As described for FF, resolved fractions of cyst fluid were generated and tested for effects on net cortisol oxidation by 11βHSD in porcine granulosa cells from large antral follicles. Fractions of cyst fluid eluted at 70-80% and 100% (v/v) methanol significantly suppressed net enzyme activities in porcine granulosa cells by up to 78±4% of the control enzyme activity ($P<0.05$; Figure 5.3). Fractions of cyst fluid eluted at 0-60% and 90% (v/v) methanol consistently lowered 11βHSD activity in granulosa cells but suppression of enzyme activities by these fractions of cyst fluid did not reach statistical significance.

5.2.4 Intrafollicular concentrations of cortisol and cortisone and cortisol:cortisone ratios in porcine antral follicles and ovarian cysts

There were no signficant differences between the mean individual cortisol and cortisone concentrations in antral follicles, or in the summed cortisol plus cortisone concentrations, irrespective of follicle diameter ($P>0.05$; Table 5.1). Therefore, the mean intrafollicular concentrations of cortisol and cortisone, and the summed cortisol plus cortisone concentrations were calculated for all antral follicles from the individual values measured in small, medium and large antral follicles. For all porcine antral follicles, the mean intrafollicular concentration of cortisol was 162±11nM, and the mean cortisone concentration in all antral follicular fluids was 28±1nM. The mean concentrations of both cortisol and cortisone were significantly lower than the corresponding hormone concentrations in ovarian cysts stated in Table 5.1 ($P<0.05$ for cortisol and $P<0.001$ for cortisone). Likewise, the mean summed cortisol plus cortisone concentration was calculated for all antral follicles (190±12nM), and was found to be significantly lower than that in ovarian cysts (Table 5.1; $P<0.001$).

Intrafollicular cortisol:cortisone ratios did not vary between small, medium and large antral follicles ($P>0.05$; Figure 5.4). As for the individual hormone concentration data presented in Table 5.1, the mean cortisol:cortisone ratios in all antral follicles was calculated, giving a value of 6.2±0.3. Cortisol:cortisone ratios

were significantly decreased in ovarian cysts (2.7±0.5) compared to the intrafollicular hormone ratio in each size class of antral follicle (Figure 5.4) and in the mean ratio for all antral follicles ($P<0.001$).

Table 5.1. Cortisol and cortisone concentrations plus summed cortisol and cortisone concentrations (nM) in in porcine FF from small, medium and large antral follicles and in porcine cyst fluid from spontaneous ovarian cysts

	Hormone concentration		Summed hormone concentrations
Follicle category	Cortisol (nM)	Cortisone (nM)	Cortisol plus cortisone (nM)
Small	159±15[a]	28±1[a]	187±15[a]
Medium	165±13[a]	25±2[a]	191±15[a]
Large	163±18[a]	30±5[a]	194±21[a]
Ovarian cyst	228±10[b]	82±9[b]	300±13[b]

Each data entry is the mean (±SEM) intra-follicular hormone concentration for 5 individual porcine FF or cyst fluid samples from different animals. Within each column, data with different superscripts differ significantly ($P<0.05$).

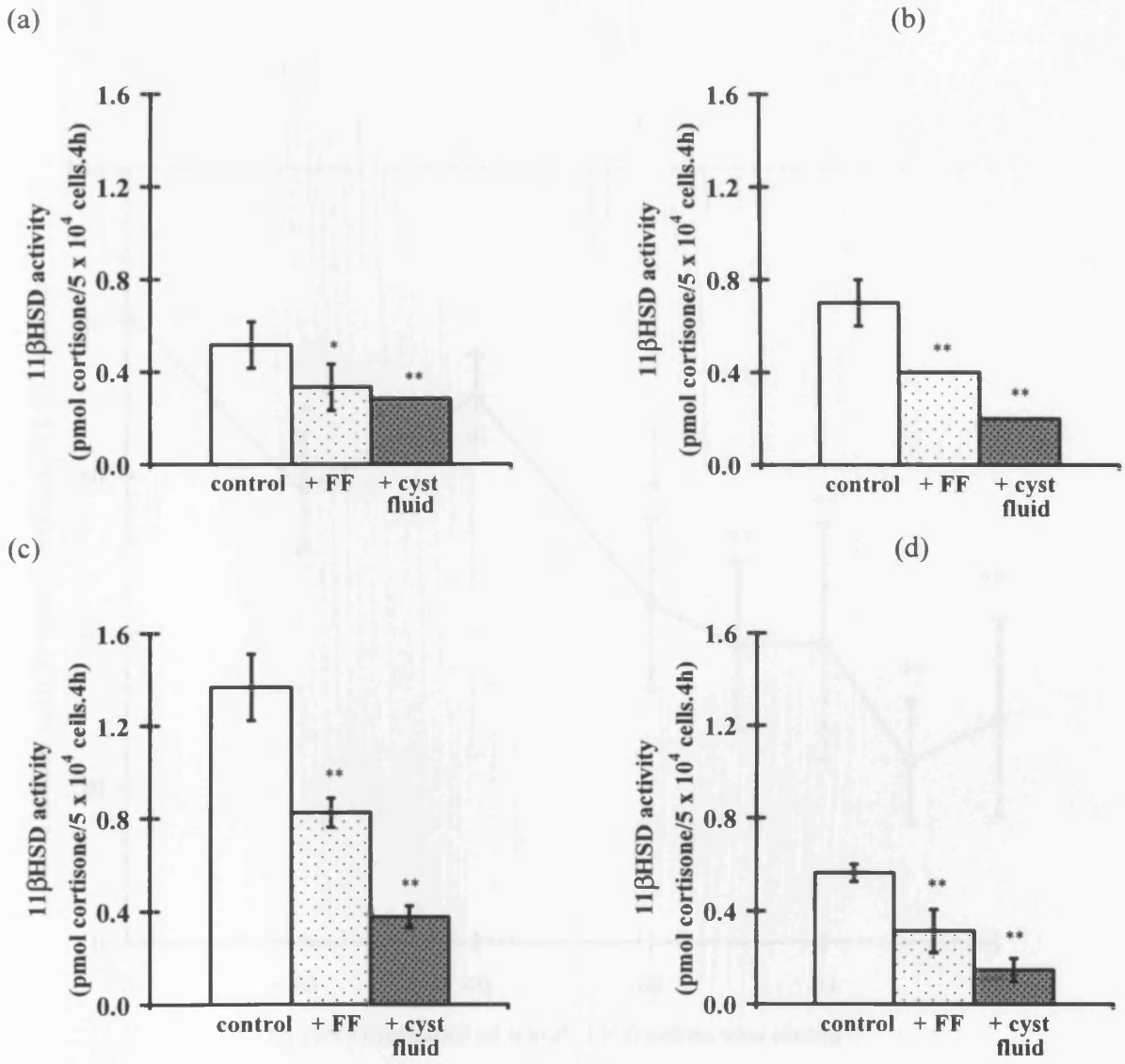


Figure 5.1. Effects of porcine FF from large antral follicles and of porcine cyst fluid from spontaneous ovarian cysts on net cortisol oxidation in porcine granulosa cells isolated from antral follicles and ovarian cysts. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone produced/5 x 10^4 viable cells.4h) for 5 granulosa cell cultures from (a) small, (b) medium and (c) large antral follicles and (d) spontaneous ovarian cysts from different animals. Each individual granulosa cell culture was incubated in medium alone (control), with 10% (v/v) porcine large antral FF (+ FF) or with 10% (v/v) porcine cyst fluid (+ cyst fluid), respectively. Each culture condition was assessed in triplicate within each of the 5 independent assays. *P<0.05 and **P<0.01 versus the respective rate of control cortisol oxidation measured in the absence of FF or cyst fluid.

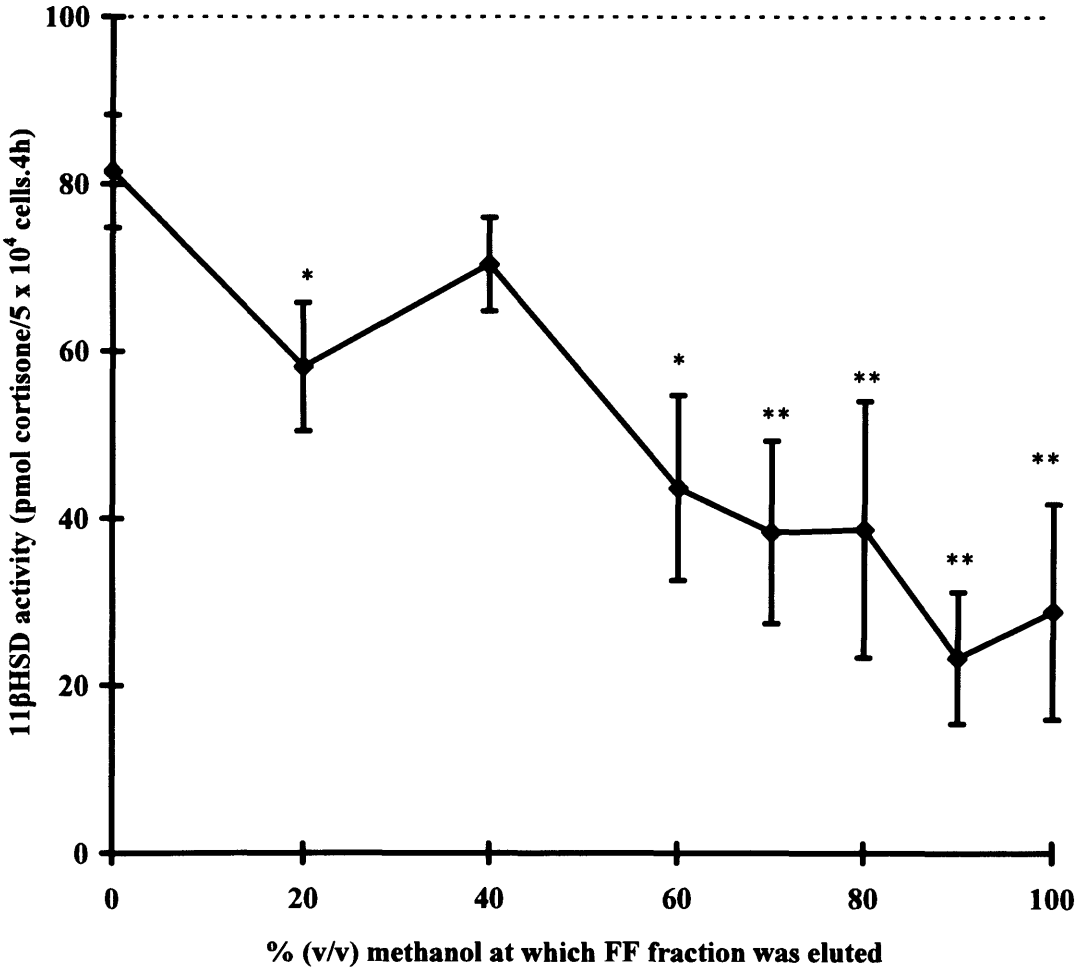


Figure 5.2. Effects of C18 fractions of porcine FF from large antral follicles on net cortisol oxidation in porcine granulosa cells from large antral follicles. Each data point represents the mean (±SEM) enzyme activity (% of control) from 5 granulosa cell cultures from large antral follicles from different animals, with a given FF fraction assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control net 11βHSD activity of 100%, which equated to 1.3±0.3pmol cortisone/5 x 10^4 viable cells.4h. *P<0.05 and **P<0.01 versus control cortisol oxidation measured in the absence of the resolved fractions of FF.

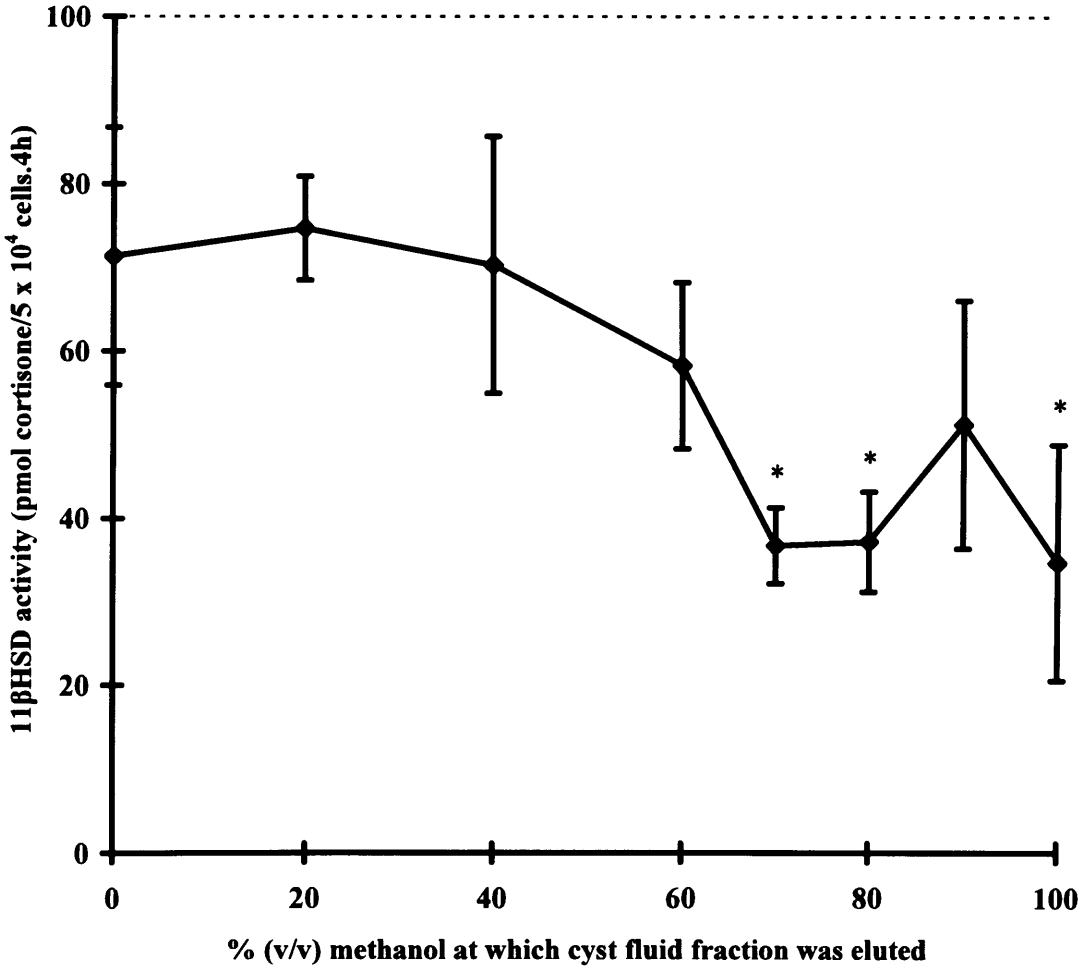


Figure 5.3. Effects of C18 fractions of porcine cyst fluid from spontaneous ovarian cysts on net cortisol oxidation in porcine granulosa cells from large antral follicles. Each data point represents the mean (±SEM) enzyme activity (% of control) for 5 granulosa cell cultures from large antral follicles from different animals, with a given cyst fluid fraction was assessed in triplicate within each of the 5 independent assays. The horizontal line indicates a control net 11βHSD activity of 100%, which equated to 1.9±0.7pmol cortisone/5 x 10^4 viable cells.4h. *$P<0.05$ versus control cortisol oxidation measured in the absence of the resolved fractions of cyst fluid.

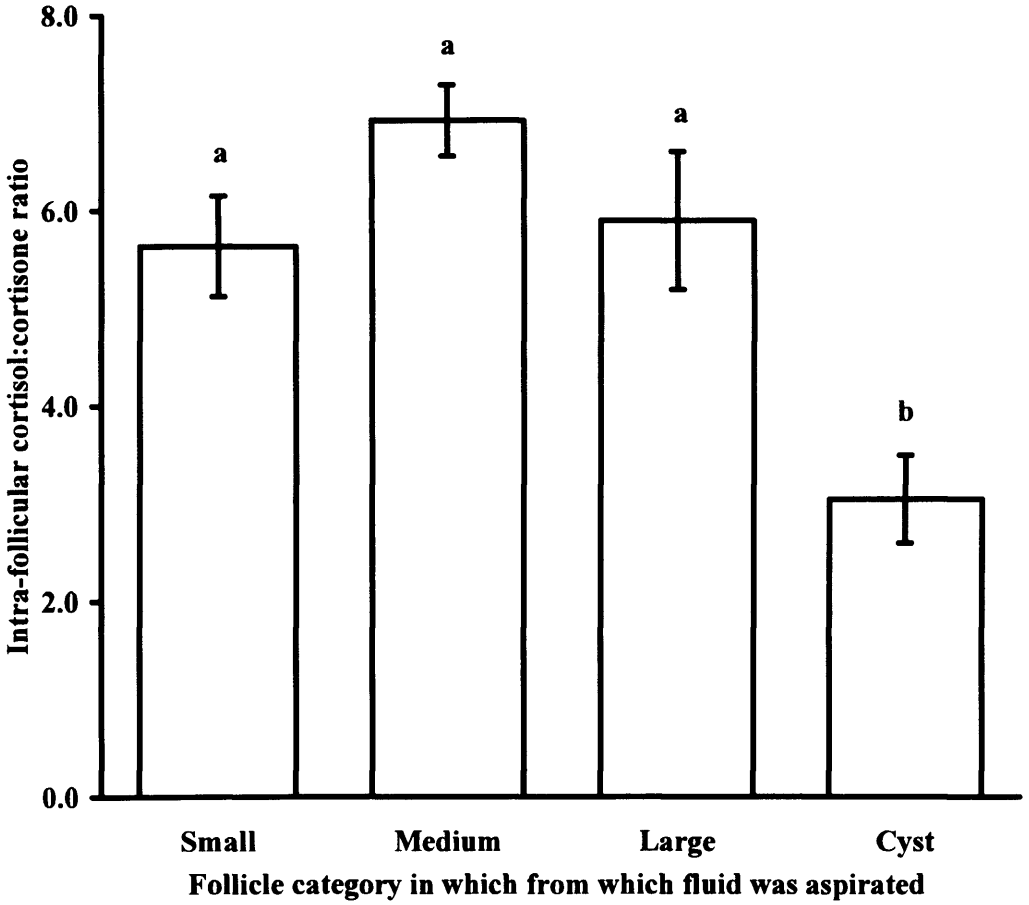


Figure 5.4. Cortisol:cortisone ratios in porcine FF from small, medium and large antral follicles and in porcine cyst fluid from spontaneous ovarian cysts. Each data point is the mean (±SEM) intra-follicular hormone ratio for 5 individual follicular fluid or cyst fluid samples from different animals. Between bars, data showing different superscripts differ significantly ($P<0.01$).

5.3 Discussion

The primary aim of this chapter was to determine the effects of porcine ovarian fluids on net cortisol oxidation by 11βHSD enzymes in mural granulosa cells. Both FF and cyst fluid were shown to suppress net 11β-DH activities in granulosa cells from antral follicles and ovarian cysts. Furthermore, the components of FF and cyst fluid eluted at ≥60% (v/v) methanol significantly inhibited net cortisol oxidation in granulosa cells from large antral follicles. Therefore, data in the present chapter indicate that the hydrophobic components of porcine ovarian fluids could act in a paracrine manner to alter 11βHSD activities in mural granulosa cells. So, in the environment of the ovarian follicle or cyst, it is possible that the enzyme inhibitors in the antral fluid could regulate cortisol metabolism by 11βHSD in the granulosa cells of the follicle or cyst wall.

FF from large antral follicles exerted similar extents of inhibition of net cortisol oxidation in all porcine granulosa cells, irrespective of follicle category. In contrast, there was a progressively increasing extent of inhibition exerted by cyst fluid on 11β-DH activities in granulosa cells from increasing follicle size. As noted in chapter 4, cyst fluid appears to contain high levels of the intrafollicular enzyme inhibitors that appear to be selective for the $NADP^+$-dependent activities of 11βHSD in renal homogenates (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). In chapter 3, the $NADP^+$-dependent activities of 11βHSD were shown to increase in granulosa cell homogenates with increasing follicle size. Thus as enzyme activities increased in cell homogenates with antral follicle growth, it could have been easier to observe enzyme suppression by the intrafollicular enzyme modulators in cyst fluid. In addition, the increasing suppression exerted by the cyst fluid on enzyme activities in granulosa cells with antral follicle growth could reflect a concurrent increase of 11βHSD1 expression in the granulosa cells with increasing follicle diameter. This possibility was also discussed in chapter 3 to offer a possible explanation for the increasing $NADP^+$-dependent activities of 11βHSD displayed in granulosa cell homogenates from antral follicles of increasing diameter.

Unlike the relatively high levels of $NADP^+$-dependent cortisol oxidation (approximately 1pmol cortisone/4h) observed in granulosa cell homogenates from large antral follicles and ovarian cysts, net cortisol oxidation in cultured granulosa cells from large antral follicles was around 2-fold higher than in granulosa cells from ovarian cysts. Notwithstanding the decreased 11βHSD activities in granulosa cells from ovarian cysts, cyst fluid exerted a high inhibitory effect (of around 70%) on enzyme activities in granulosa cells from both large antral follicles and ovarian cysts. This suggests that cyst fluid contains relatively high levels of the hydrophobic 11βHSD1 inhibitors and/or that these compounds are more potent than the enzyme modulators in large antral FF

Although cyst fluid suppressed 11βHSD activities in granulosa cells to a greater extent than FF, the resolved inhibitory fractions of cyst fluid and FF suppressed enzyme activities in granulosa cells to comparable extents. Similar findings were observed in chapter 4, for which rat kidney homogenates were used as a source of 11βHSD activity. In renal homogenates, a decreasing extent of inhibition of net 11βHSD activities was brought about by FF from follicles of increasing size, with the greatest suppression exerted by cyst fluid. However after the use of C18 column chromatography, the inhibitory fractions eluted from FF and cyst fluid were shown to inhibit 11βHSD activities in renal homogenates to similar extents. This appears to suggest that the endogenous 11βHSD1 inhibitors of porcine ovarian fluid as a whole have a greater suppressive effect, in total, than the 11βHSD1 inhibitor(s) eluted in isolated fractions of that fluid.

The findings in this chapter indicate that 11βHSD1 inhibitors in porcine ovarian fluids could influence the metabolism of cortisol by 11βHSD in porcine mural granulosa cells. In turn, the levels of cortisol metabolism in the granulosa cells may be reflected by the intrafollicular cortisol:cortisone ratios; a potential index of 11βHSD activities *in vivo*. With the increasing net 11β-DH activities observed in the mural granulosa cells from antral follicles of increasing diameter, one might expect concurrently decreasing cortisol:cortisone ratios in the FF of growing antral follicles. Since significantly decreased 11β-DH activities were displayed in

the granulosa cells from ovarian cysts, one could predict an increased intrafollicular cortisol:cortisone ratio within the antral fluid of ovarian cysts (relative to large antral FF). The findings reported in this chapter however, show that the predicted changes were not observed in the intrafollicular cortisol:cortisone ratios of antral follicles and ovarian cysts. Cortisol:cortisone ratios did not change in FF with increasing antral follicle size. Moreover, in the fluid from ovarian cysts there were significantly decreased (rather than increased) cortisol:cortisone ratios. This may simply be because the capacity of isolated mural granulosa cells to oxidise cortisol in primary culture may not reflect the abilities of the same cells inside intact ovarian follicles to metabolise cortisol *in vivo*. Thus it would be useful to culture intact porcine ovarian follicles and harvest the granulosa cells at defined stages of follicle growth before measuring intracellular 11βHSD activities. Additionally, the intrafollicular cortisol:cortisone ratios could be measured in the ovarian fluids.

Given that 11βHSD enzymes alter the balance of cortisol and cortisone at the cytoplasmic receptors, any changes in intracellular cortisol metabolism may be too subtle to be reflected in the extracellular/intrafollicular cortisol:cortisone ratio. The results of studies conducted in this thesis indicate that the picomolar conversion of cortisol to cortisone was observed in primary granulosa cell cultures. Thus intrafollicular cortisol:cortisone ratios in the antral follicles may in fact be predominantly governed by the nanomolar concentrations of glucocorticoids in the systemic circulation. Hence cortisol which is being interconverted with cortisone at a site(s) outside of the ovarian follicle, could enter the circulation and reach the ovary through the ovarian artery. *In vivo*, cortisol metabolism occurring in the granulosa cells in ovarian follicles may be magnitudes of order lower than levels of cortisol metabolism occurring in non-ovarian tissues. If the blood entering and leaving the ovary was potentially transporting glucocorticoids to and from the ovarian follicle, this may determine the intrafollicular cortisol:cortisone ratios. Thus perfusion studies could be conducted in cultured porcine ovarian follicles with known concentrations of glucocorticoids entering the follicles. Cortisol:cortisone ratios could then be measured in the FF and in the fluid leaving the follicle, and this could be

compared to the levels of net cortisol oxidation occurring in the granulosa cells of those cultured ovarian follicles.

The altered cortisol:cortisone ratios observed in cyst fluid, which were counter to expectations, may be associated with COD. The lowered cortisol:cortisone ratios in ovarian cysts could reflect the low 11-KSR activities of 11βHSD1 enzymes in the cells of the cyst. Interestingly, a defect in the 11-KSR activity of 11βHSD1 can manifest as apparent cortisone reductase deficiency (ACRD; described in section 1.4.4) in women, which can be associated with anovulatory infertility (Phillipou and Higgins, 1985; Phillipov *et al.*, 1996; Jamieson *et al.*, 1999).

Studies in this chapter also reported significantly increased absolute concentrations of cortisol in cyst fluid, which could also be linked to COD. The higher glucocorticoid concentrations observed in cyst fluid may be a reflection of high concentrations of glucocorticoids in the systemic circulation. Increased glucocorticoid production could be induced by elevated CRH and ACTH synthesis. Interestingly, the pathogenesis of COD has been linked to an activation of the HPA axis, as described in section 1.2.4. Studies have reported that high plasma CRH, ACTH or glucocorticoid levels could be associated with a decrease in GnRH or LH pulse frequency and/or release (Scholten and Liptrap, 1978; Dobson *et al.*, 1988; Williams *et al.*, 1990; Dobson *et al.*, 2000; Ribadu *et al.*, 2000). Thus a decrease in the preovulatory LH surge, due to the activation of the HPA axis, may result in the formation of anovulatory spontaneous ovarian cysts from preovulatory follicles.

High concentrations of glucocorticoids in cyst fluid may also be related to the signs of altered steroidogenesis observed in porcine ovarian cysts (Jana *et al.*, 2005), given that glucocorticoids were shown to modify ovarian steroidogenesis (Hsueh and Erickson, 1978; Michael *et al.*, 1993b). In addition, glucocorticoids can inhibit prostaglandin production (Goppelt-Struebe, 1997), pro-inflammatory cytokine production (Telleria *et al.*, 1998), HAS-2 expression (Stuhlmeier and Pollaschek, 2004). All of the events affected by glucocorticoids appear to play

crucial roles in ovulation (reviewed by Richards (2005)). Thus the increased glucocorticoid concentrations in cyst fluid may suppress the ability of ovarian cysts to rupture and undergo ovulation. In addition, glucocorticoids could exert anti-apoptotic effects in granulosa cells from ovarian cysts (Sasson *et al.*, 2001), which may thereby prevent the regression of these cysts.

Finally, it is possible that the cortisol:cortisone ratios in porcine ovarian fluid may be regulated by 11βHSD enzymes in the theca or cumulus cells, or the oocyte, rather than just the mural granulosa cells. This could account for the unchanging cortisol:cortisone ratios in FF with antral follicle growth, despite the increasing 11βHSD activities in mural granulosa cells and decreasing levels of intrafollicular enzyme inhibitors. This may also explain the significantly decreased cortisol:cortisone ratios in ovarian cysts. Within the ovarian follicle, the porcine oocyte and/or the accompanying cumulus cells would be obvious candidates in which to investigate the expression and activity of the 11βHSD enzymes, and this forms the focus of the next chapter.

Chapter 6

11βHSD Enzymes in Porcine Cumulus-Oocyte Complexes and Effects of the Intrafollicular Enzyme Modulators on Enzyme Activities

6.1 Background

The findings presented in the preceding chapter raised the possibility that 11βHSD enzymes may be expressed in cells in the interior of porcine ovarian follicles and cysts other than the mural granulosa cells. Given that the porcine cumulus granulosa cells and oocyte are located in the antrum of an ovarian follicle, where they would be exposed to the endogenous 11βHSD1 modulators contained in the antral follicular/cyst fluid, these cells would be strong candidates in which to investigate the expression and activities of 11βHSD enzymes.

Benediktsson *et al.* (1992) were the first to document the expression of high levels of 11βHSD1 mRNA in the preovulatory oocytes from ovaries of rats in proestrus. 11βHSD1 protein was subsequently shown to be expressed in the oocytes contained in human primordial, primary and preovulatory follicles, with no detectable expression of 11βHSD2 (Ricketts *et al.*, 1998). Smith *et al.* (2000) later published the presence of mRNAs encoding 11βHSD1, but not 11βHSD2, in oocytes and cumulus cells from human MII COCs, i.e. human COCs in which the oocyte was arrested in the metaphase stage of the second meiotic division.

These published reports of 11βHSD expression in oocytes are interesting given that glucocorticoids have been documented to have various effects on oocyte maturation, which depend on the species being studied. As discussed in section 1.3.3.1, cortisol has been shown to induce oocyte maturation in a number of species of fish (Kime *et al.*, 1992; Petrino *et al.*, 1993; Mugnier *et al.*, 1997; Pinter and Thomas, 1999; Milla *et al.*, 2006; Mishra and Joy, 2006). Andersen (2003) determined the effects of glucocorticoids on IVM of mouse oocytes. The results of his work indicated that the rate of GVBD did not change in oocytes cultured with increasing concentrations of dexamethasone, although dexamethasone did appear to decrease the proportion of oocytes progressing from GVBD to MII. In porcine oocytes, Yang *et al.* (1999) documented negative effects of glucocorticoids on nuclear maturation. Both dexamethasone and cortisol decreased GVBD in porcine oocytes and COCs. When dexamethasone and the glucocorticoid receptor (GR) antagonist, RU-486, were co-incubated with porcine

oocytes, RU-486 abolished the inhibitory effects of dexamethasone on GVBD. Thus dexamethasone appeared to be exerting effects on porcine oocyte maturation through the GR. One possible mechanism of dexamethasone action is through effects on the expression of MPF (maturation promoting factor; Masui and Market, 1971), which mediates the resumption of meiosis in the oocyte. MPF is a protein complex that consists of CDK-1 (p34cdc2) and cyclin B1 (Gautier *et al.*, 1990; de Vantery *et al.*, 1997; Kanatsu-Shinohara *et al.*, 2000). In pig COCs, dexamethasone was shown to decrease levels of cyclin B1, without affecting CDK-1, resulting in diminished levels of total MPF (Chen *et al.*, 2000). This could account for the negative effects of glucocorticoids on porcine oocyte maturation that were reported by Yang *et al.* (1999).

IVM studies have documented the positive effects of FF from large antral follicles on the maturation of cultured porcine oocytes (Naito *et al.*, 1988; Naito *et al.*, 1989; Yoshida *et al.*, 1992; Funahashi and Day, 1993). More recently in the pig, the effects of FF on porcine oocyte maturation were associated with the size of the follicle from which fluid was aspirated. Porcine COCs cultured in the presence of FF from large antral follicles had higher rates of cumulus expansion and nuclear maturation than COCs cultured with FF from small or medium antral follicles (Vatzias and Hagen, 1999; Yoon *et al.*, 2000; Algriany *et al.*, 2004). This could be, in part, attributed to the presence of follicular fluid-meiosis activating sterol (FF-MAS) in the fluid of preovulatory follicles, which has been shown to induce mammalian oocyte maturation (Byskov *et al.*, 1999). Though FF-MAS is a hydrophobic compound that is present in FF it was potentially discounted as a potential candidate for the main intrafollicular 11βHSD1 inhibitor in the chapter 4 of this thesis. This was because the highest levels of FF-MAS would be expected to occur in FF from large antral follicles however the studies reported in chapter 4 indicated that the lowest levels of endogenous 11βHSD1 modulators appear to be present in porcine large antral FF.

CBX, which is an established inhibitor of the activities of both 11βHSD1 and 11βHSD2 as discussed in sections 1.2.2 and 1.4.5, has been shown to induce the

maturation of oocytes enclosed in rat ovarian follicles (Sela-Abramovich *et al.*, 2006). The proposed mechanism by which CBX was thought to exert effects on oocyte maturation was through the blocking of gap junctions (Webb *et al.*, 2002; Sela-Abramovich *et al.*, 2006), an effect which CBX appeared to exert in cultured rat granulosa cells after 30 minutes. Since the rat oocyte has been shown to express 11βHSD1 (Benediktsson *et al.*, 1992), CBX may also exert inhibitory effects on 11βHSD1 activity in the oocyte. Thus the dual ability of CBX to suppress 11βHSD1 activity and block gap junctions may be associated with an increase in oocyte maturation. Given that the endogenous enzyme inhibitors have the capacity to modulate the activities of 11βHSD1, it would be worthy to investigate whether the intrafollicular enzyme inhibitors exerted any effects on porcine oocyte maturation.

The aims of this chapter were therefore:

1. To identify whether the 11βHSD enzymes are expressed in porcine COCs from antral follicles.

2. To measure 11βHSD activities in COCs and denuded oocytes (DOs) at different stages of oocyte maturation.

3. To determine if the endogenous inhibitors of 11βHSD1 present in FF or cyst fluid can affect 11βHSD activities in COCs and DOs.

4. To establish whether the inhibitors of 11βHSD1 in cyst fluid, or the resolved fractions of cyst fluid, affect the maturation of cultured porcine COCs.

6.2 Results

6.2.1 Expression of the 11βHSD enzymes in porcine COCs

For RT-PCR analysis, 50-100 compact COCs were pooled after isolation from small and medium antral follicles (of 2-3 and 4-7mm in diameter, respectively), and then lysed in buffer. 200-300ng of the total RNA extracted from the pooled COCs were reverse transcribed in a 20μl reaction. Thereafter, 2-3μl of the

resulting cDNA were amplified in a 50µl PCR reaction mix. Details of the RT-PCR protocol, including the PCR cycling conditions, are given in section 2.7.2.

Porcine granulosa cells from large antral follicles and porcine liver and kidney tissue were used as positive controls for all primer sets. Positive control primers run for each sample were 18S, β-actin and GAPDH. At the RT step, negative controls were prepared by incubating total RNA samples without reverse transcriptase enzyme (RT-) and further negative controls were prepared at the PCR step by adding DNase/RNase-free water in place of cDNA for each individual primer set (W).

Figure 6.1 represents a typical RT-PCR product gel in which the cell- or tissue-specific 11βHSD1 and 11βHSD2 mRNA expression profile was demonstrated in 3 individual sows. The figure shows that neither 11βHSD1 nor 11βHSD2 mRNA transcripts were observed in COCs. An mRNA transcript for 11βHSD1 was observed in porcine liver (Li) and in porcine granulosa cells from large antral follicles (GC(L)), and a transcript for 11βHSD2 was detected from porcine kidney (K) and from porcine granulosa cells from large antral follicles (GC(L)). Negative control RT(-) samples, incubated with *hsd11b1* or *hsd11b2* primers, showed that no genomic DNA was present in any of the cell or tissue samples. The negative water control indicated that the PCR equipment or reagents were not contaminated with any nucleic acids.

Figure 6.2 depicts a representative RT-PCR product gel depicting the cell- or tissue-specific 18S, β-actin or GAPDH mRNA expression in 3 individual sows. Bands resulting from the amplification of gene products of 18S, β-actin and GAPDH were identified in all cells/tissues on all gels. This confirmed the integrity of the total RNA isolated from all cell and tissue samples, and the detection of β-actin and GAPDH products indicated that the RT step had been successful for cell/tissue samples. It could be noted however, that very faint bands were detected for β-actin and GAPDH expression in COCs, indicating that there were may have been low levels of cDNA generated from the COC samples. These

levels of cDNA may have therefore have been insufficient to detect 11βHSD expression in COCs. Negative control RT(-) samples were run for all positive control primers in all cell/tissue samples and confirmed the absence of genomic DNA in all samples. The negative water control indicated that the PCR equipment or reagents were not contaminated with any nucleic acids.

In terms of identifying mRNA transcripts for 11βHSD enzyme isoforms in porcine COCs, the results of this RT-PCR study appeared inconclusive. Given that the expression of 11βHSD1 was shown in rat and human oocytes as described above, the determination of net 11βHSD activities in COCs was continued with as stated in aim 2 of this chapter.

6.2.2 11βHSD activities in compact and expanded porcine COCs

Net 11βHSD activities were measured in freshly isolated compact and expanded porcine COCs, and in the DOs isolated from compact and expanded COCs (protocol details are given in section 2.7.3). Compact COCs were collected from FF aspirated from small (2-3mm in diameter) and medium (4-7mm diameter) antral follicles and expanded COCs were isolated from large antral follicles (≥8mm diameter). DOs were isolated by vortexing expanded or compact COCs with 0.1% (w/v) hyaluronidase (detailed in section 2.7.1). Compact COCs and the DOs isolated from compact COCs were incubated in groups of 5, whereas expanded COCs and the DOs obtained from expanded COCs were each cultured individually. All groups of COCs or DOs were incubated for 24 hours and enzyme activities were determined via a radiometric conversion assay over the 24 hours. As enzyme activities in compact and expanded DOs and COCs increased linearly with oocyte or COC number, levels of net cortisol oxidation for each type of COC were expressed per single oocyte/COC. Studies conducted by other members of our research group have indicated that the 11-KSR activities are not displayed in porcine COCs, thus only effects on 11β-DH activities (net cortisol oxidation) will be discussed hereafter.

Low 11β-DH activities were observed in freshly isolated compact COCs and in the DOs prepared from compact COCs ($P>0.05$; Figure 6.3). Levels of net cortisol oxidation in intact expanded COCs were approximately 10-fold higher than those in compact COCs ($P<0.001$). The DOs isolated from expanded COCs displayed lower net 11β-DH activities than those in the intact expanded COCs ($P<0.05$) however net 11β-DH activities in DOs from expanded COCs were around 7-fold higher than those in DOs isolated from compact COCs ($P<0.001$).

6.2.3 11βHSD activities in porcine oocytes from IVM studies

Subsequent to IVM studies, individual groups of COCs were denuded and viewed under a light microscope. As stated in section 2.7.6, the chromatin and microtubules were stained in a proportion of oocytes from IVM studies and viewed under a fluorescence microscope to identify whether nuclear maturation had taken place. When viewed under the light microscope, all oocytes which did not appear to extrude a polar body showed no indications of nuclear maturation when stained and viewed under the fluorescent microscope (Figure 6.4a). Conversely, a metaphase plate was seen in every fluorescently-stained oocyte for which a polar body had been observed under the light microscope (Figure 6.4b).

11βHSD activities were compared in oocytes that had or had not matured *in vitro* from compact COCs. As 11βHSD activities could not be assessed in IVM oocytes that had been fixed and stained, and given that metaphase plates had been detected in all fluorescently-stained oocytes exhibiting polar bodies, the visual assessment of oocytes under the light microscope (i.e. the presence/absence of a polar body) was used to determine whether or not oocytes had reached MII. Net cortisol oxidation was significantly higher in the oocytes that had extruded a polar body following IVM, compared to oocytes which had not ($P<0.01$; Figure 6.5). The difference between the net 11βHSD activities in the two groups of oocytes was approximately 12-fold.

IVM studies were conducted over a 48-hour incubation period, after which a 24-hour radiometric conversion assay was carried out to measure 11βHSD activities in oocytes that had or had not matured in culture. Thus the length of time in which oocytes were placed into primary culture may have potentially affected basal enzyme activities in the IVM oocytes. To address this possibility, the levels of net cortisol oxidation in oocytes from IVM studies were compared with enzyme activities in the DOs from freshly isolated compact and expanded COCs presented in Figure 6.3. On comparison of the data in Figures 6.3 and 6.5, it could be noted that 11β-DH activities in the oocytes that had not appeared to mature in culture were similar to enzyme activities in DOs prepared from freshly isolated compact COCs ($P>0.05$). Likewise, levels of net cortisol oxidation in porcine oocytes that appeared to have undergone IVM were comparable to those in DOs from freshly isolated expanded COCs ($P>0.05$).

6.2.4 Effects of porcine ovarian fluids on 11βHSD activities in porcine COCs DOs from COCs

The fluids from large antral follicles and ovarian cysts were tested for effects on 11βHSD activities in freshly isolated compact and expanded COCs, and in the DOs from each category of COC. The fluids from large antral follicles and ovarian cysts were selected as they appeared to contain the lowest and highest levels of the endogenous hydrophobic 11βHSD1 inhibitors, respectively. In addition, sufficient volumes of each ovarian fluid could be obtained from a single large antral follicle or ovarian cyst on an ovary from individual animals.

Any inhibitory effects of the ovarian fluids on enzyme activities in COCs or DOs were compared to the effects of two known inhibitors of 11βHSD activities, CBX and GA. Both CBX and GA appear to non-selectively inhibit the activities of both cloned 11βHSD enzyme isoforms. GA is the more potent of the two inhibitors and has been reported to preferentially inhibit 11β-DH activities of the 11βHSD enzymes (Monder *et al.*, 1989), while CBX has been documented to suppress both 11β-DH and 11-KSR activities of 11βHSD1 (Stewart *et al.*, 1990).

FF from large antral follicles had no significant effects on enzyme activities in intact compact COCs ($P>0.05$; Figure 6.6). Cyst fluid, however, significantly inhibited 11βHSD activities in intact compact COCs by 45±16%, relative to control enzyme activity ($P<0.05$). CBX and GA both suppressed 11β-DH activities in compact COCs ($P<0.01$). As for intact compact COCs, net oxidation of cortisol in the DOs from compact COCs was not altered by large antral FF ($P>0.05$; Figure 6.7) whereas cyst fluid inhibited 11βHSD activities in DOs from compact COCs by 62±5%, compared to control rates of cortisol oxidation ($P<0.01$). CBX and GA both inhibited net cortisol oxidation by 11βHSD to a similar extent than cyst fluid; this was by 65±8% and 65±7% of control 11βHSD activities, respectively ($P<0.01$).

FF from large antral follicles and cyst fluid both significantly altered net cortisol oxidation by 11βHSD in intact expanded COCs (Figure 6.8). Cyst fluid had a greater suppressive effect and decreased net 11βHSD activity by 75±3% of control enzyme activity, compared to 57±10% inhibition by FF from large follicles ($P<0.01$). CBX and GA also exerted suppressive effects on net cortisol oxidation in expanded COCs, compared to control activities ($P<0.01$). FF from large antral follicles, cyst fluid, CBX and GA each exerted significant suppressive effects on 11βHSD activities in DOs from expanded COCs ($P<0.01$; Figure 6.9).

6.2.5 Effects of porcine cyst fluid, and resolved fractions thereof, on IVM of oocytes from compact porcine COCs

As discussed above, the beneficial effects of FF from large antral follicles on porcine oocyte maturation have been reported by several IVM studies. Thus far, any effects of porcine cyst fluid on oocyte maturation have not been reported. Therefore in this study, cyst fluid and the resolved fractions thereof were tested for effects on IVM of porcine COCs. The IVM protocol was as described in section 2.7.4. Two separate sets of resolved fractions of cyst fluid were generated; the first set came from cyst fluid fractions which were eluted at 0-20% (v/v) methanol and then pooled (this will hereafter be referred to as the "hydrophilic fractions of cyst fluid"). The second set of cyst fluid fractions were eluted at 70-

100% (v/v) methanol and subsequently (these will described as the "hydrophobic fractions of cyst fluid"). Therefore, only the most hydrophilic or hydrophobic components of cyst fluid were tested for effects on oocyte maturation. After 48 hours in culture with cyst fluid, or the pooled hydrophilic or hydrophobic fractions of cyst fluid, each group of COCs were denuded and viewed under the light microscope, as detailed in section 2.7.5. As described above, the presence of a polar body under the light microscope was deemed to be an indication that oocytes had undergone *in vitro* maturation and reached MII.

The data presented in Table 6.1 shows that the total proportion of oocytes (from 4 independent IVM studies) reaching MII was significantly higher in the presence of porcine cyst fluid (64%) than in the control group (26%, total χ^2 = 49.312; P<0.001). The resolved hydrophobic fractions of fluid induced greater rates of oocyte maturation (44%) than the control group (22%, χ^2 = 46.372; P<0.001; Table 6.2). Likewise, the proportion of porcine oocytes reaching MII was greater in the presence of the hydrophilic fractions of porcine cyst fluid (68%) than in the control group.

Table 6.1. The effects of porcine cyst fluid from spontaneous ovarian cysts on the total numbers (and percentages) of oocytes that matured in culture (extruded a polar body)

Condition	Number of oocytes	- polar body	+ polar body	$\chi 2$ value	P value
control	163	121 (74%)	42 (26%)	49.312	P<0.001
+ cyst fluid	181	66 (36%)	115 (64%)		

Each group of compact COCs was incubated in maturation medium alone (control), or in the presence of 10% (v/v) cyst fluid (+ cyst fluid). The table shows the total number of oocytes (calculated from 4 separate IVM studies) in the control and cyst fluid-treated groups, followed by the total number of oocytes that did not extrude a polar body (- polar body) and the total number that did extrude a polar body (+ polar body) in that particular treatment group. The numbers in brackets represent the percentage of oocytes in each treatment group that did not or did extrude a polar body. The P-value and level of significance indicated by the Chi-squared test was assessed using the total data values of the 4 independent studies presented in this table.

Table 6.2. The effects of the resolved fractions of porcine cyst fluid from spontaneous ovarian cysts on the total numbers (and percentages) of oocytes that matured in culture (extruded a polar body)

Condition	Number of oocytes	- polar body	+ polar body	χ^2 value	P value
control	108	84 (78%)	24 (22%)		
+ hydrophilic fractions	112	36 (32%)	76 (68%)	46.372	P<0.001
+ hydrophobic fractions	103	58 (56%)	45 (44%)		

Each group of compact COCs was incubated in maturation medium alone (control), or in the presence of 10% (v/v) of the pooled hydrophilic or hydrophobic fractions of cyst fluid. The table shows the total number of oocytes (calculated from 4 separate IVM studies) in the control and the treated groups, followed by the total number of oocytes that did not extrude a polar body (- polar body) and the total number that did extrude a polar body (+ polar body) in that particular treatment group. The numbers in brackets represent the percentage of oocytes in each treatment group that did not or did extrude a polar body. The P-value and level of significance indicated by the Chi-squared test was assessed using the total data values of the 4 independent studies presented in this table.

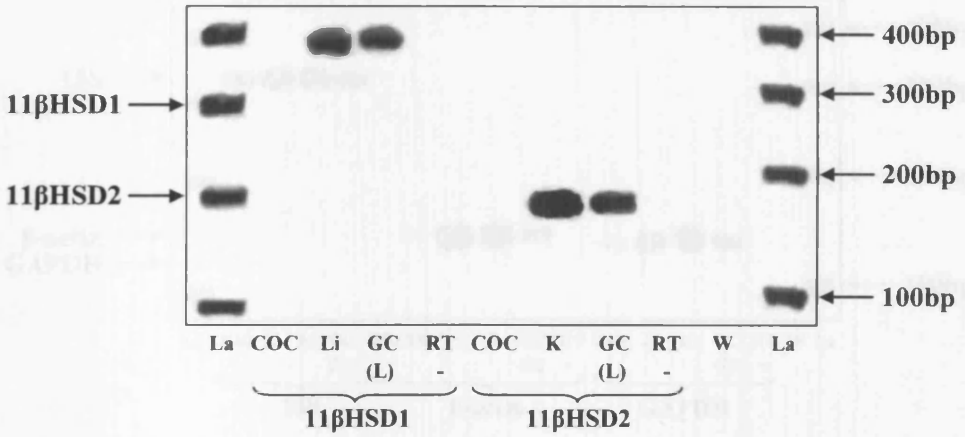


Figure 6.1. 11βHSD1 and 11βHSD2 mRNA expression in porcine granulosa cells, and porcine liver and kidney tissue. Amplicons generated using primers specific for 11βHSD1 and 11βHSD2 cDNA were of the correct, predicted sizes (394bp and 221bp, respectively). Gel lanes are marked as follows: La = ladder, COC = cumulus-oocyte complexes, Li = liver tissue, K = kidney tissue, GC(L) = granulosa cells from large antal follicles. RT(-) samples were prepared by incubating total RNA extracted from COCs with DNase/RNase-free water in the place of reverse transcriptase. W represents the water control, in which DNase/RNase-free water was amplified in place of cDNA.

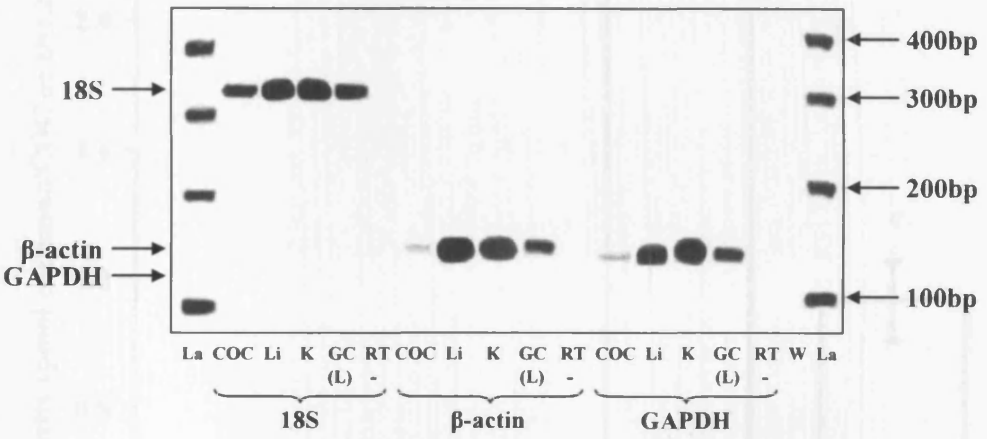


Figure 6.2. 18S, β-actin and GAPDH expression in porcine granulosa cells, and porcine liver and kidney. Amplicons generated for 18S, β-actin and GAPDH were of the predicted sizes (315bp, 169bp and 162bp respectively). Gel lanes are marked as follows: La = ladder, COC = cumulus-oocyte complexes, Li = liver tissue, K = kidney tissue, GC(L) = granulosa cells from large antal follicles. RT(-) samples were prepared by incubating total RNA extracted from COCs with DNase/RNase-free water in the place of reverse transcriptase. W represents the water control, in which DNase/RNase-free water was amplified in place of cDNA.

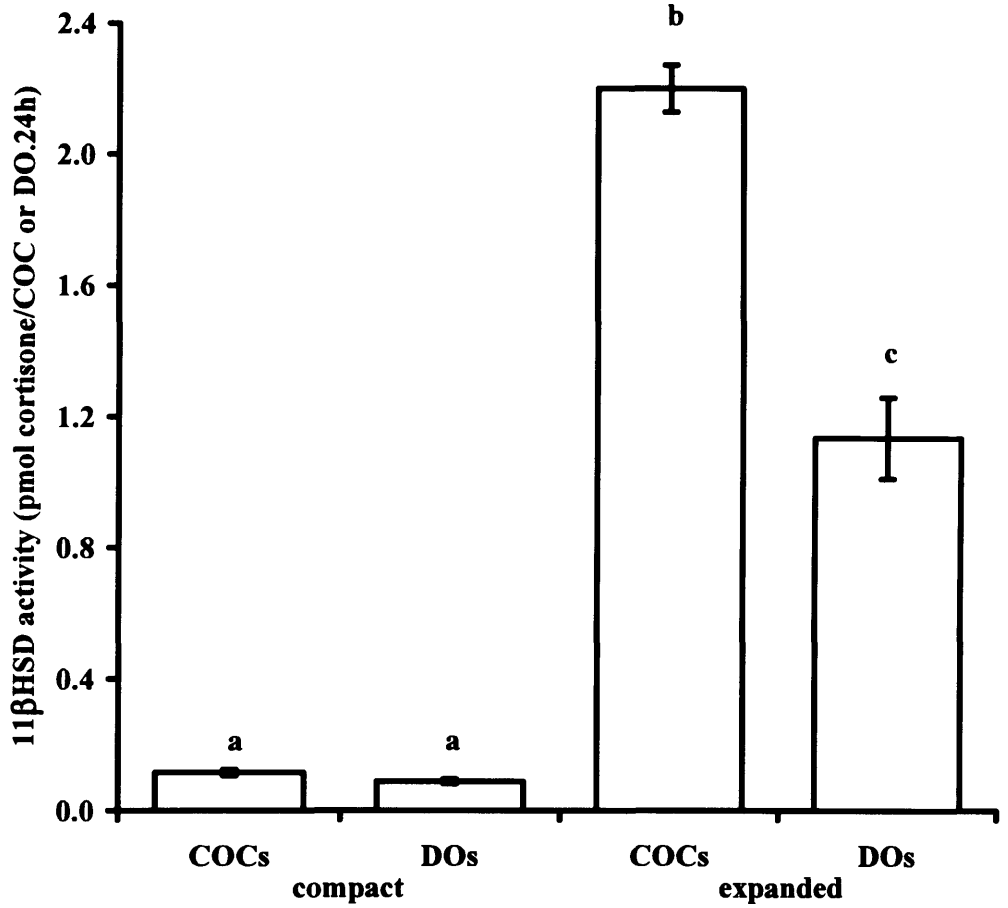
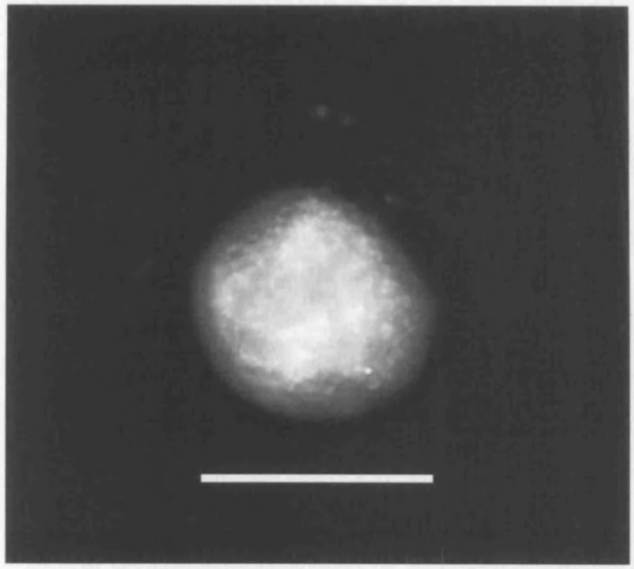


Figure 6.3. Net cortisol oxidation in DOs from compact COCs, in intact compact COCs, in DOs from expanded COCs and in intact expanded porcine COCs. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone/COC or DO.24h) for 5 independent assays of COC or DO groups. Between bars, data showing different superscripts differ significantly ($P<0.05$).

(a)



(b)

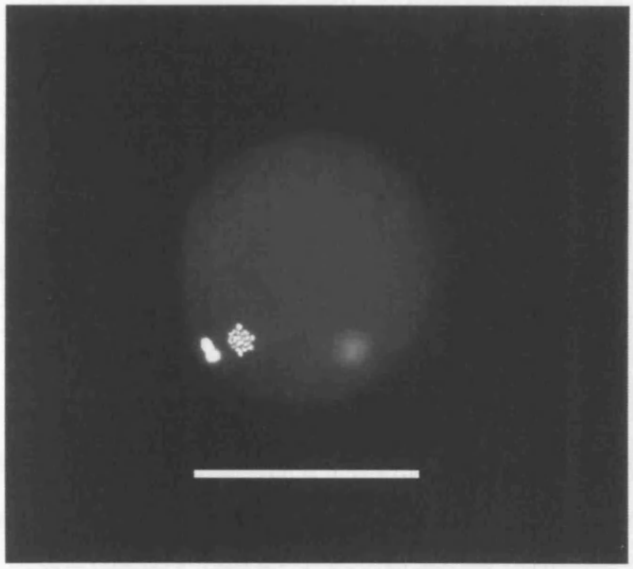


Figure 6.4. Images of oocytes collected from IVM studies that were stained with PI and α-tubulin. Image (a) shows an oocyte for which a polar body was not observed and (b) shows an oocyte for which a polar body was exhibited. These images were representative of every oocyte, without or with a polar body, that were visualised under a fluorescent microscope from 4 independent IVM studies. Scale bar represents 100μm.

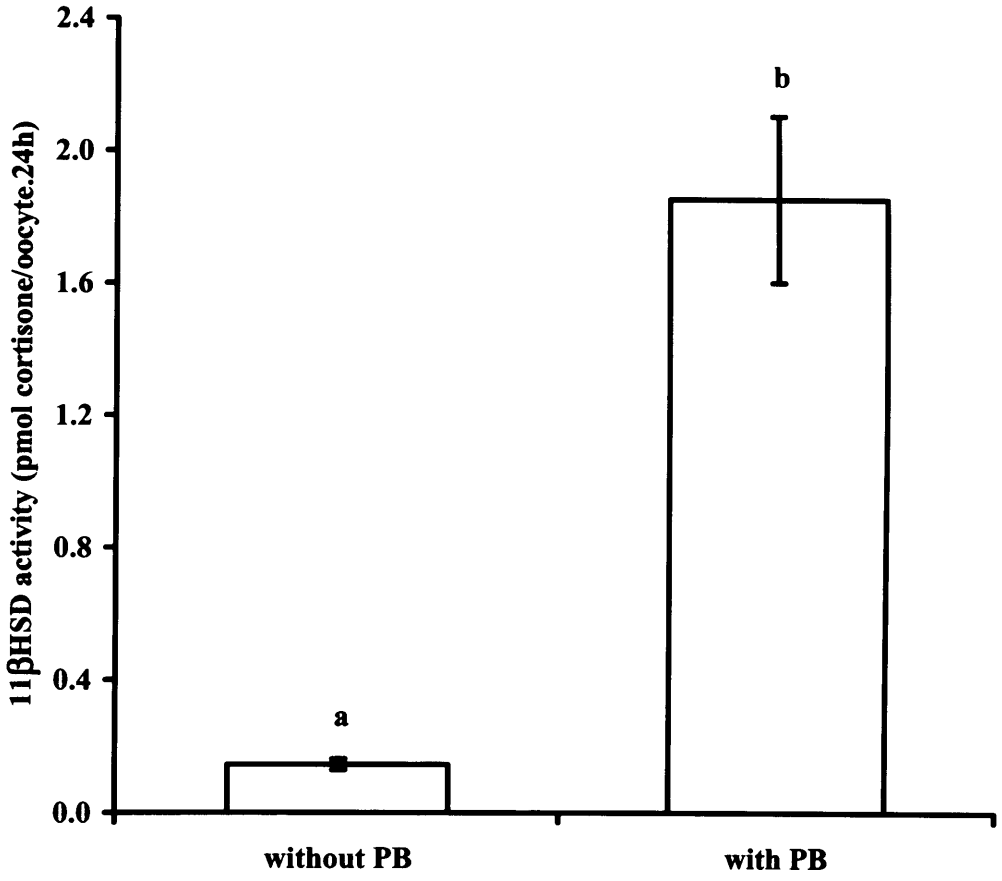


Figure 6.5. Net cortisol oxidation in oocytes without/with polar bodies after IVM. Each data point for the oocytes collected from IVM studies represents the mean (±SEM) enzyme activity (pmol cortisone/oocyte.24h) for 5 independent assays of groups of 5 oocytes. Between bars, data showing different superscripts differ significantly ($P<0.001$).

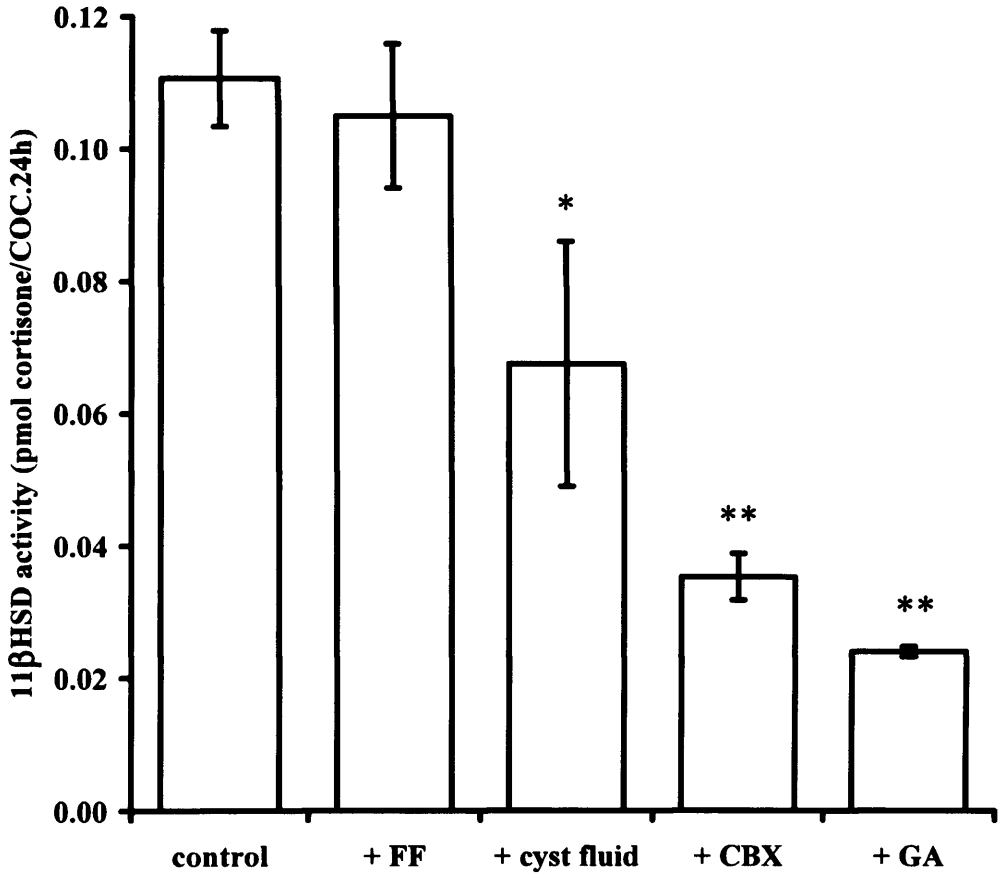


Figure 6.6. Effects of porcine FF from large antral follicles and of porcine cyst fluid from spontaneous ovarian cysts on net cortisol oxidation in compact porcine COCs. COCs were incubated in medium alone (control), or in the presence of 10% (v/v) porcine large antral FF (+ FF), 10% (v/v) porcine cyst fluid (+ cyst fluid), 10% (v/v) 10μM CBX (+ CBX) or 10% (v/v) 10μM GA (+ GA), respectively. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone/COC.24h) for 5 independent assays of COC groups. *P<0.05 and **P<0.01 versus respective control cortisol oxidation measured in the absence of porcine FF, cyst fluid, CBX or GA.

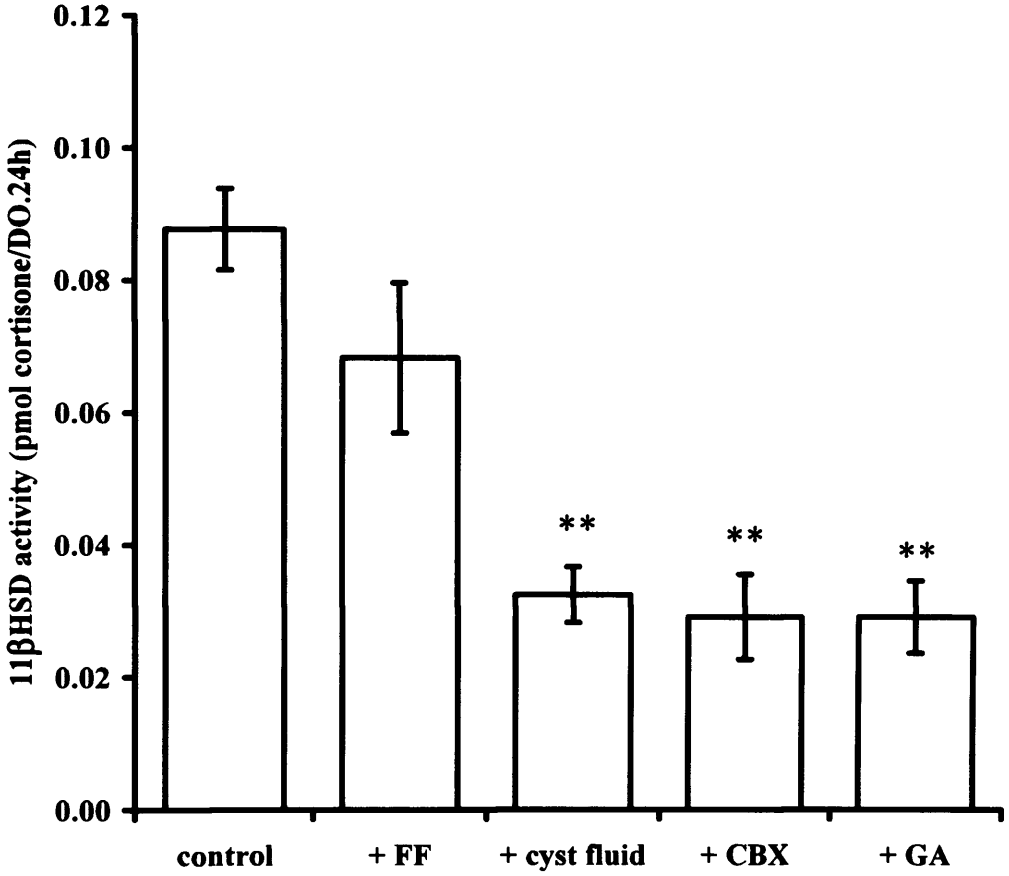


Figure 6.7. Effects of porcine FF from large antral follicles and porcine cyst fluid from spontaneous ovarian cysts on net cortisol oxidation in DOs from compact porcine COCs. DOs were incubated in medium alone (control), or in the presence of 10% (v/v) porcine large antral FF (+ FF), 10% (v/v) porcine cyst fluid (+ cyst fluid), 10% (v/v) 10μM CBX (+ CBX) or 10% (v/v) 10μM GA (+ GA), respectively. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone/DO.24h) for 5 independent assays of DO groups. **P<0.01 versus respective control cortisol oxidation measured in the absence of porcine FF, cyst fluid, CBX or GA.

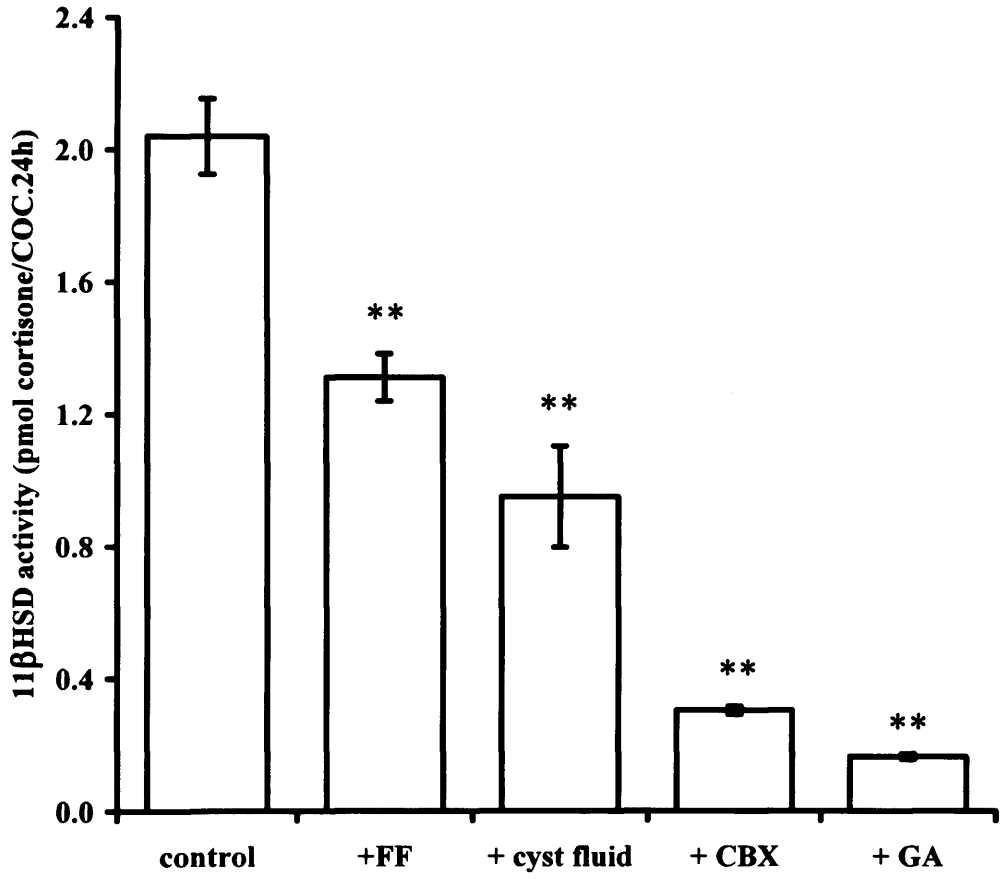


Figure 6.8. Effects of porcine FF from large antral follicles and of porcine cyst fluid from spontaneous ovarian cysts on net cortisol oxidation in expanded porcine COCs. COCs were incubated in medium alone (control), or in the presence of 10% (v/v) porcine large antral FF (+ FF), 10% (v/v) porcine cyst fluid (+ cyst fluid), 10% (v/v) 10μM CBX (+ CBX) or 10% (v/v) 10μM GA (+ GA), respectively. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone/COC.24h) for 5 independent assays of COCs. **P<0.01 versus respective control cortisol oxidation measured in the absence of porcine FF, cyst fluid, CBX or GA.

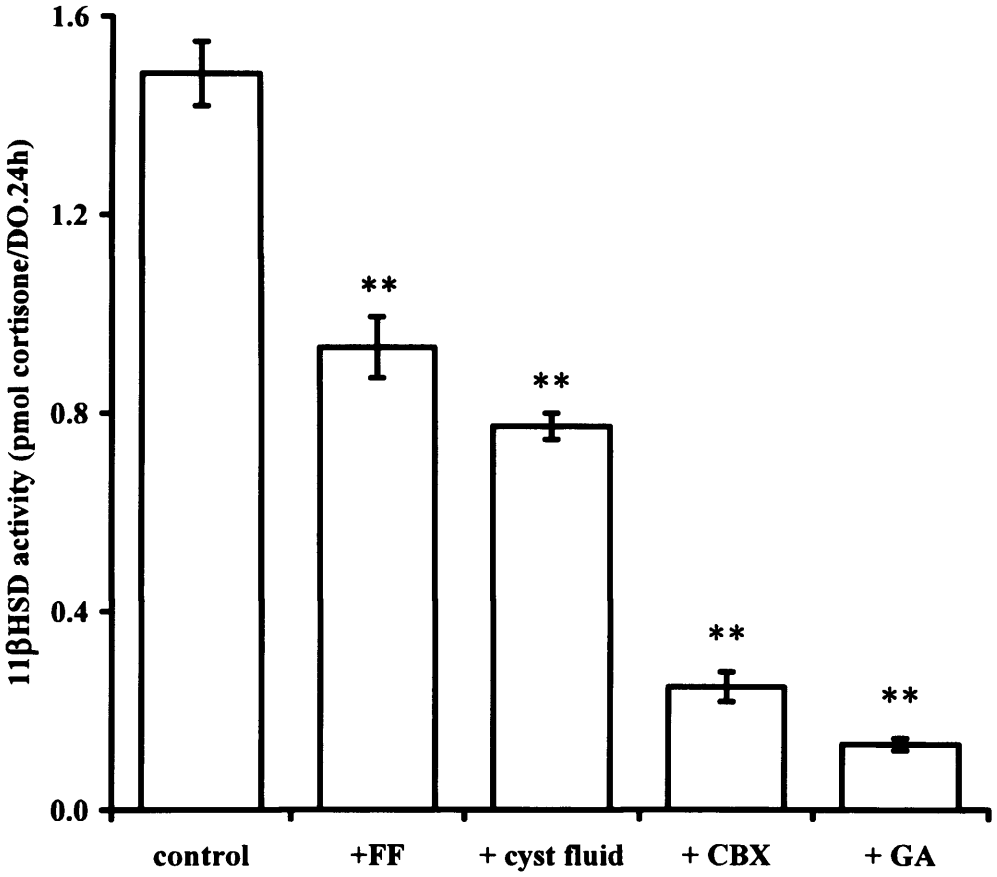


Figure 6.9. Effects of porcine FF from large antral follicles and of porcine cyst fluid from spontaneous ovarian cysts on net cortisol oxidation in DOs from expanded porcine COCs. DOs were incubated in medium alone (control), or in the presence of 10% (v/v) porcine large antral FF (+ FF), 10% (v/v) porcine cyst fluid (+ cyst fluid), 10% (v/v) 10μM CBX (+ CBX) or 10% (v/v) 10μM GA (+ GA), respectively. Each data point represents the mean (±SEM) enzyme activity (pmol cortisone/DO.24h) for 5 independent assays of oocytes. **P<0.01 versus respective control cortisol oxidation measured in the absence of porcine FF, cyst fluid, CBX or GA.

6.3 Discussion

In this chapter, the expression and activities of the 11βHSD enzymes were investigated in porcine COCs and in DOs isolated from COCs. Furthermore, the effects of the intrafollicular 11βHSD1 inhibitors in porcine ovarian fluids were tested for effects on 11βHSD activities in COCs and DOs and for effects on porcine oocyte maturation.

RT-PCR assays attempting to identify 11βHSD1 or 11βHSD2 expression in porcine compact COCs were deemed to be inconclusive. In porcine liver, kidney and granulosa cells, which served as positive controls, 11βHSD1 or 11βHSD2 cDNA products of the anticipated size were generated, confirming the reliability of the primers and indicating that the RT and PCR steps had been successful in these samples. The positive control primers (18S, β-actin and GAPDH) used in this expression study amplified gene products of the anticipated sizes in liver, kidney, granulosa cells and COCs, further indicating the integrity of the RT-PCR process. Though housekeeping genes such as β-actin and GAPDH are likely to be constitutively expressed, it has been documented in the literature that levels of gene expression could vary depending on the tissue or cells of interest and/or culture treatments and conditions. An example which could apply to ovarian follicle cells is the apparent decrease in GAPDH expression during hypoxia (Zhong and Simons, 1999). The occurrence of hypoxia has been associated with follicle growth (see review by Neeman *et al.* (1997)). Despite this, recent studies have analysed the expression of 18S, β-actin or GAPDH during oocyte maturation in two mammalian species (cow and buffalo). In these species, the expression of 18S, β-actin or GAPDH was shown to be stable and did not change during IVM (Bettegowda *et al.*, 2006; Aswal *et al.*, 2007).

Although 18S, β-actin and GAPDH products were observed in COCs, the β-actin and GAPDH gene products in compact COCs appeared as very faint bands, indicating potentially low levels of cDNA generated from the COC samples. This suggests that the method used for the initial extraction of total RNA from porcine COCs may not have been optimal. It was also possible that a proportion of the

mRNA had degraded before the RT step, though the levels of 18S expression in COCs appeared to confirm the integrity of the total RNA in the COC samples. In addition, a proportion of cDNA in the COC samples might have had degraded between the RT and PCR steps. If the final quantity of cDNA in the COC samples was insufficient, then the detection of 11βHSD enzyme expression may not have been possible without the optimisation of the RT-PCR protocol. Additionally, nested PCR reactions could be employed, which would involve the use of a second set of 11βHSD1 and 11βHSD2 primers to amplify the gene products of the first 11βHSD primer sets. Future work in this area could also involve ascertaining whether 11βHSD expression occurred in both cell types of the porcine COC, that is, in the cumulus cells and the oocyte. Furthermore, it would be interesting to compare 11βHSD enzyme expression in the porcine compact COC with that in the expanded COC using real-time PCR.

Despite an inability to detect 11βHSD1 or 11βHSD2 expression using RT-PCR, western blotting studies currently being carried out in our laboratory have indicated that 11βHSD1 protein is expressed in porcine COCs. This data is not yet sufficiently robust to include in this chapter. In light of this preliminary expression data, and given that 11βHSD1 was shown to be expressed in preovulatory rat oocytes (Benediktsson *et al.*, 1992) and in human oocytes from all stages of follicular development (Ricketts *et al.*, 1998; Smith *et al.*, 2000), the measurement of 11βHSD activities in COCs was proceeded with. The detection of net cortisol oxidation in freshly isolated compact and expanded COCs indicated that at least one of the 11βHSD enzymes must be expressed and, moreover, be functional, in porcine COCs. On-going work by other members of our laboratory strongly suggests that reductase activities are not observed in porcine COCs. These findings indicate that the 11βHSD enzyme(s) in porcine COCs predominantly, if not exclusively, act as dehydrogenases as was shown to be the case in porcine mural granulosa cells in chapter 4.

When the cumulus cells were removed from COCs, the DOs from compact COCs had comparable 11βHSD activities with intact compact COCs and DOs from

expanded COCs had moderately lower enzyme activities than intact expanded COCs. These observations indicate that the oocyte is the source of the majority of 11βHSD activity in the compact COC, with relatively low enzyme activities in the cumulus cells. To determine the proportion of the 11βHSD activity observed in an intact COC that could be attributed to the cumulus cells, 11βHSD activities would need to be measured in isolated cumulus cells. As there appears to be exclusive 11βHSD1 expression in human cumulus cells (Smith *et al.*, 2000), it would be worth attempting to measure both 11β-DH and 11-KSR activities in the cumulus cells. One might then be able to determine what proportion of the 11βHSD activity in an intact COC could be attributed to the cumulus cells and oocyte, individually.

The 11β-DH activities were significantly higher in intact expanded COCs than in compact COCs. Likewise, enzyme activities were significantly higher in the DOs from expanded COCs than in DOs from compact COCs. IVM studies showed that levels of cortisol oxidation were around 12-fold greater in oocytes from compact COCs that had matured *in vitro* than in oocytes that did not mature in culture, or in oocytes from freshly isolated compact oocytes (likely to be in the GV stage). All of the above findings suggest that oocyte maturation is associated with an increase in net cortisol oxidation by 11βHSD, observed in both COCs and in DOs. High dehydrogenase activities were also displayed in the mural granulosa cells from porcine large antral follicles (indicated by the findings of studies in chapter 3). Since glucocorticoids appear to have negative effects on the nuclear maturation of porcine oocytes (Yang *et al.*, 1999), the high levels of cortisol metabolism observed in the oocyte and the granulosa cells of porcine large antral follicles could thus aid in protecting the preovulatory oocyte from the potentially detrimental effects of glucocorticoids on oocyte maturation. Considering the anovulatory status of ovarian cysts, it would be interesting to investigate the level of 11β-DH activities in the COCs of ovarian cysts.

Expanded COCs were likely to have experienced an LH surge *in vivo* before being collected from the fluid of preovulatory follicles. As mentioned above,

levels of net cortisol oxidation were significantly increased in freshly isolated expanded COCs relative to compact COCs. In IVM studies, the presence of LH in the maturation medium serves to mimic the *in vivo* LH surge to induce cumulus expansion and the resumption of meiosis in oocytes *in vitro*. In the IVM study in this thesis chapter, high 11β-DH activities were observed in the *in vitro* matured COCs. Therefore, the effects of LH, either *in vivo* or *in vitro*, could be linked to the increased 11βHSD activities observed in freshly isolated expanded COCs and *in vitro* matured COCs. Indeed, hCG- and LH-treatment have each been reported to upregulate 11βHSD1 expression in rat granulosa cells (Tetsuka *et al.*, 1999a; Tetsuka *et al.*, 1999b). One caveat to this suggestion, however, is that the mechanisms by which LH exerts effects on the oocyte are still unknown. The majority of studies in various of species report that neither the oocyte nor cumulus cells express the LH receptor (LHR) (Peng *et al.*, 1991; van Tol *et al.*, 1996; Eppig *et al.*, 1997; Shimada *et al.*, 2003). Some studies however have detected LHR mRNA in the oocytes (Patsoula *et al.*, 2001; Patsoula *et al.*, 2003) and cumulus cells (Bukovsky *et al.*, 1993; Chen *et al.*, 1994; Mattioli *et al.*, 1998; Shimada *et al.*, 2003). If the cumulus cells and/or the oocyte lack functional LHRs, any effects of LH on 11βHSD expression in the oocyte would have to be mediated by the mural granulosa cells, on which LHRs were shown to be expressed in preovulatory follicles (Nimrod *et al.*, 1977). To expand on this, the binding of LH to its receptors on the granulosa cells could, in turn, increase intracellular cAMP levels. If cAMP in the mural granulosa cells could be transported to the cumulus cells and the oocyte, via gap junctions, then the actions of LH in the mural granulosa cells could potentially influence 11βHSD enzyme expression in the COC. Interestingly, the expression of both 11βHSD1 and 11βHSD2 has been reported to be increased by intracellular cAMP (Sun *et al.*, 1998; Gout *et al.*, 2006). Thus it would be worthy to determine the effects of LH and/or cAMP on 11βHSD expression and activity in the oocyte.

As described in section 1.2.2, LH can upregulate PGHS-2 expression in the cumulus cells to increase prostaglandin production prior to ovulation (Morris and Richards, 1993). Recent work by our group showed that the pharmacological inhibition of prostaglandin production, in turn, decreased the 11β-DH and 11-KSR

activities of 11βHSD1 in human granulosa-lutein cells (Jonas *et al.*, 2006). This implies that in preovulatory follicles, the stimulation of prostaglandin production by the LH surge may also increase 11βHSD1 activities. Thus the stimulation of prostaglandin production in the cells of the preovulatory ovarian follicle may be linked to the increased 11β-DH activities observed in the mural granulosa cells and/or COC of these follicles. Cortisol has been shown to inhibit the intraovarian synthesis of prostaglandins (Goppelt-Struebe, 1997). Since prostaglandins are involved in the mechanisms of both cumulus expansion and ovulation (Espey, 1980; Richards, 2005), the increased metabolism of cortisol in the cells of large antral follicle could serve to alleviate the potentially negative effects of cortisol on prostaglandin production.

In terms of receptor-mediated effects of cortisol, the glucocorticoid hormone has been shown to inappropriately activate the mineralocorticoid receptor (MR). Thus the expression of 11βHSD2 in mineralocorticoid target tissues appears to inactivate cortisol to prevent excessive activation of the MR (Edwards *et al.*, 1988; Funder *et al.*, 1988). MR expression was recently documented in the bovine oocyte with increasing MR expression following oocyte maturation (Robert *et al.*, 2000) hence it is possible that the activation of the MR could play a role in the development of the mammalian oocyte. The expression of 11βHSD2 has not been reported in the mammalian oocyte thus far. High levels of net cortisol oxidation observed in expanded porcine COCs would appear to mimic the 11β-DH activity of 11βHSD2 to protect the MR from the potential effects of intracellular cortisol in the oocyte. In order to test the potential role of the MR in oocyte maturation, the presence of MR mRNA would need to be ascertained in porcine oocytes using RT-PCR. Furthermore, real-time PCR could be employed to compare levels of MR expression in compact *versus* expanded porcine oocytes. To determine any effects of mineralocorticoids on porcine oocyte maturation, porcine COCs could be incubated in the presence of increasing concentrations of aldosterone, deoxycorticosterone (DOC) or a synthetic pure mineralocorticoid, such as fludrocortisone. Thereafter, effects on GVBD and the progression of oocytes to MII could be compared in COCs incubated the presence or absence of the above steroids. This may indicate whether mineralocorticoid action has the potential to

affect oocyte maturation. An alternative study could involve blocking the MR, using a compound such as spironolactone, to determine whether oocyte maturation could occur in the absence of MR activation.

The afore-mentioned study by Benediktsson *et al.* (1992) detected high levels of 11βHSD1 mRNA in preovulatory rat oocytes. Interestingly, in the preovulatory oocytes from a number of mammalian species, such as pigs, cows, sheep and mice, the transcription of new mRNA is blocked after the resumption of meiosis and only recommences in the embryo (see review by Telford *et al.* (1990)). The high expression of 11βHSD1 reported in maturing rat oocytes may therefore reflect a role for the 11βHSD1 enzyme in the late stages of oogenesis or possibly after fertilisation, in the zygote or early embryo. Thus further work could involve conducting RT-PCR and/or real-time PCR assays to measure levels of 11βHSD1 enzyme expression in preovulatory porcine COCs, and comparing these to levels of enzyme expression in the porcine zygote, and at various stages of porcine pre-implantation embryo development (e.g. in the 2-, 4-, 8-, 16-cell porcine morula and blastocyst).

In the preceding chapters of this thesis, the intrafollicular enzyme modulators in FF from porcine antral follicles were shown to significantly alter $NADP^+$-dependent 11βHSD1 activities in rat kidney homogenates (chapter 4) and inhibit net cortisol oxidation in mural porcine granulosa cells (chapter 5). FF from large antral follicles, however, could not affect 11β-DH activities in compact COCs or in the DOs from compact COCs. It was noteworthy, however, that large antral FF could affect net cortisol oxidation in expanded COCs and DOs from expanded oocytes; this result will be addressed later. As control 11βHSD activities were approximately 12-fold higher in expanded COCs and DOs from expanded COCs, it could simply be more feasible to see a suppressive effect of large antral FF on enzyme activities in expanded COCs and their DOs, and less so in compact COCs and their DOs.

Unlike large antral FF, porcine cyst fluid significantly suppressed net cortisol oxidation both in compact and expanded COCs, and in the DOs isolated from compact and expanded COCs. Thus porcine cyst fluid appeared to contain higher levels of endogenous enzyme inhibitors and/or more potent inhibitors of 11βHSD1, consistent with the findings of chapters 4 and 5. CBX and GA consistently exerted greater inhibitory effects of 11βHSD activities, both in COCs and DOs than did either large antral FF or cyst fluid. While CBX and GA can inhibit the activities of both 11βHSD1 and 11βHSD2, the intrafollicular enzyme inhibitors appear to selectively suppress the activity of 11βHSD1. The greater extent of enzyme inhibition exerted by CBX and GA may thus suggest that a proportion of the net 11β-DH activities in COCs and DOs could be attributed to 11βHSD2.

The findings of IVM studies conducted in this chapter showed that cyst fluid was able to induce the nuclear maturation of porcine oocytes and also exerted effects on cumulus cell expansion. This positive effect on oocyte maturation was also observed in the presence of only the hydrophobic components of cyst fluid, which were likely to contain the endogenous 11βHSD1 inhibitors and possibly FF-MAS. Published work has thus far documented the beneficial effects of FF from large antral follicles on the *in vitro* maturation of porcine oocytes (Naito *et al.*, 1988; Naito *et al.*, 1989; Yoshida *et al.*, 1992; Funahashi and Day, 1993). FF from large antral follicles was shown to suppress 11β-DH activities in expanded porcine COCs and the DOs from expanded COCs in findings reported in this chapter. Likewise, CBX was shown to inhibit net cortisol oxidation in all porcine COCs and DOs. CBX was also able to induce rat oocyte maturation (Sela-Abramovich *et al.*, 2006) which linked to effects of cyst fluid on oocyte maturation.

In the study by Sela-Abramovich *et al.* (2006), it was suggested that CBX could induce oocyte maturation by inhibiting gap junction communication. CBX was shown to block gap junctions between cultured rat granulosa cells after 30 minutes, and also induced germinal vesicle breakdown (GVBD) in a proportion of rat oocytes after 2 hours. The short length of time within which CBX affected gap

junction communication and oocyte maturation suggests that CBX potentially exerts post-translational effects on both biological functions, rather than altering gene transcription. Previous work conducted by our research group showed that CBX could inhibit 11β-DH activities in renal homogenates during a 1-hour incubation period (Thurston *et al.*, 2002). Therefore, CBX probably suppresses 11βHSD activities through a post-translational effect on the enzyme, almost certainly by binding to the active site of the enzyme as does 18β-GA (Irie *et al.*, 1992). This raises the possibility that post-translational effects of CBX on gap junctions and 11βHSD activity could both be involved in the induction of oocyte maturation by CBX. In studies conducted in chapter 4, and in published studies, porcine ovarian fluids from antral follicles and ovarian cysts also displayed acute suppressive effects on enzyme activities in renal homogenates (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). Given that porcine large antral FF and cyst fluid both appear to exert positive effects on oocyte maturation, the endogenous 11βHSD1 inhibitors in these fluids could induce porcine oocyte maturation through post-translational effects on gap junctions and/or 11βHSD activity. Thus further work could involve determining whether the intrafollicular enzyme inhibitors could also block gap junctions in the porcine COC. This may be ascertained by incubating COCs with the hydrophobic fractions of cyst fluid before assessing the movement of a fluorescently labelled dye, such as FITC-labelled dextran, through the gap junctions using fluorescence microscopy. This method was used by Webb *et al.* (2002) to determine the effects of CBX on the gap junction communication in mouse COCs.

If the intrafollicular enzyme inhibitors in ovarian fluids could block gap junctions, it would still be unknown as to whether the suppression of 11βHSD activities or the inhibition of gap junctions, or indeed both processes, could induce porcine oocyte maturation. Thus, to elucidate whether the inhibition of 11βHSD alone could affect oocyte maturation, 11βHSD expression could be knocked down in the porcine oocyte using small interfering RNA (siRNA) to suppress gene transcription, or morpholino anti-sense oligonucleotides to prevent protein expression (MAOs). The effect of knocking down of 11βHSD expression alone could then be assessed to see if there are any potential changes in porcine oocyte

maturation. Additionally, oocytes lacking 11βHSD expression could then be incubated with CBX to determine whether CBX could still induce oocyte maturation in the absence of 11βHSD expression. If CBX could induce oocyte maturation in oocytes lacking 11βHSD, this could indicate that 11βHSD is not involved in the induction of oocyte maturation by CBX. However, if CBX was unable to induce oocyte maturation in the absence of 11βHSD expression in the oocyte, this would suggest that the 11βHSD enzyme is a mediator of the effects of CBX on oocyte maturation.

Although the hydrophobic components of porcine cyst fluid, known to contain inhibitors of 11βHSD1 activity, increased the rates of porcine oocyte maturation, high rates of porcine oocyte maturation also occurred in the presence of the hydrophilic components of cyst fluid. The positive effects of the hydrophilic components of cyst fluid may simply be attributed to LH in the ovarian fluid, which is known to induce oocyte maturation. Moreover, there was LH present in the culture medium thus the combined effects of LH in the cyst fluid and in the culture medium could have increased rates of oocyte maturation. The concentrations of LH in the cyst fluid alone, or in the culture medium containing cyst fluid, could have been evaluated by conducting a bioassay (e.g. by assessing the cyst fluid, or the culture medium containing cyst fluid, for effects on intracellular cAMP synthesis or testosterone concentrations in rat Leydig cells.

The significantly higher rates of oocyte maturation occurring in the presence of cyst fluid, or the hydrophilic and hydrophobic components thereof, may be attributable to the low percentages (on average 20%) of oocyte maturation in the control groups. The seemingly low rates of oocyte maturation in the control groups could be a reflection of the culture medium selected for use in IVM studies conducted in the present chapter. Many IVM culture media are known to contain FF, which is often used as a component of maturation medium that has been shown to induce IVM. However, FF was not added to the culture medium in IVM studies of the present chapter. This was due to the fact if both FF and cyst fluid were present in the oocyte culture medium, it would not have been possible to

deduce which of the ovarian fluids could be exerting effects on oocyte maturation. Since the focus of IVM studies in this chapter was to test the effects of cyst fluid and the components thereof on oocyte maturation, FF had to be excluded from the culture medium. The necessary lack of FF in the culture medium may have potentially compromised rates of oocyte maturation in the control groups of oocytes, however, it allowed the potential effects of cyst fluid or cyst fluid components, alone, to be assessed on porcine oocyte maturation in the treated oocyte groups.

Overall, the results from this chapter indicate that the dehydrogenase activities of 11βHSD increase in COCs and DOs coincident with oocyte maturation. This correlates with the increasing 11β-DH activities observed in granulosa cells with antral follicle growth, and may be linked to negative effects of glucocorticoids on oocyte maturation (Yang *et al.*, 1999). FF from large antral follicles could only suppress the 11βHSD activities in expanded COCs or DOs from expanded COCs. Cyst fluid, however, suppressed net cortisol oxidation in compact and expanded oocytes/COCs and the hydrophobic components of cyst fluid were also shown to increase the rates of porcine oocyte maturation. This raises the possibility that the inhibition of 11βHSD activities in the porcine COC is a mechanism by which oocyte maturation could be enhanced.

Chapter 7

General Discussion

The goal of this thesis was to ascertain whether cortisol metabolism by the 11βHSD enzymes in the ovarian follicle could play a role in folliculogenesis and/or altered antral follicle development in cystic ovarian disease (COD). To achieve this goal, the following five research areas were investigated:

1. The expression of the 11βHSD isoforms in the mural granulosa cells and COCs of porcine ovarian follicles.

2. The activities of the 11βHSD enzymes in the mural granulosa cells and COCs in porcine antral follicles of defined sizes.

3. The levels of the intrafollicular 11βHSD1 modulators in FF during porcine antral follicle growth, and in the fluid from spontaneous ovarian cysts.

4. The effects of the 11βHSD1 modulators in FF and cyst fluid on 11βHSD activities in porcine mural granulosa cells and COCs.

5. The effects of 11βHSD1 modulators in porcine cyst fluid on the IVM of porcine oocytes.

The links between stress, cortisol and reproduction in female pigs have been investigated by several research groups. The onset of puberty in prepuberal pigs appears to be induced by acute stress (du Mesnil du Buisson and Signoret, 1962; Martinat *et al.*, 1970). Conversely, the majority of findings suggest that chronic stress (environmental, physical, physiological or psychological) impairs reproductive performance in pigs (see review by (von Borell *et al.*, 2007)). However, Turner and Tilbrook (2006) reported that the reproductive abilities of sows are not susceptible to single and repeated acute stressors.

A number of different studies have employed various methods to determine whether pigs show a stress response prior to slaughter at the abattoir. One such study measured mean serum cortisol concentrations of pigs from 4 different farms prior to slaughter at an abattoir by electrical stunning (Weeding *et al.*, 1993). At the abattoir, pigs had significantly higher mean serum concentrations of cortisol, which were in the range of 32-50 ng/ml ($P<0.001$). This suggested that the pigs might have experienced an endocrine response to stress at the abattoir. Another research group investigated the effects of transport to the abattoir as a potential cause of stress prior to slaughter (Geverink *et al.*, 1998). Forty-one groups of slaughter pigs were transported in a lorry for 25 minutes while another 43 groups of sows were loaded onto a lorry that then remained stationary for 25 minutes. Salivary cortisol concentrations were significantly higher in the transported pigs than the pigs kept in the stationary lorry. Perez *et al.* (2002) conducted research into the stress responses of pigs kept at a Spanish abattoir for three different lairage times (0, 3 and 9 hours). Mean plasma cortisol concentrations were measured in blood samples collected from 150 pigs. The highest plasma cortisol concentrations (99ng/ml) were measured in pigs that were slaughtered immediately on arrival at the abattoir (0 hour group). These glucocorticoid levels were significantly higher than the plasma hormone concentrations detected in animals that were kept for 3 hours prior to slaughter (78 ng/ml; $P<0.05$) but not significantly different to animals retained for 9 hours (89 ng/ml). Therefore, this study concluded that a shorter period of lairage of a few hours could reduce the amount of stress exhibited by pigs at slaughter. On the other hand, no lairage or an excessively long lairage period could compromise animal welfare.

Another possible marker of stress is acute phase protein (APP), concentrations of which were evaluated in the serum of pigs transported over 24 or 48 hours (Pineiro *et al.*, 2007). Mean serum APP levels were higher in the pigs transported for either the 24- or 48-hour periods than the same pigs one month later while at rest. In another study, APP concentrations were measured in pigs from which blood samples were taken before transport, on arrival at an abattoir (following 12 hours of transport), and at the slaughter-line (after 6 hours in lairage). In the latter study, levels of serum APP were not significantly different before transport or immediately on arrival at the abattoir, but the levels of APP at the slaughter-line were significantly higher than those in pigs before transport and on immediate arrival. All of the above results therefore appear to show that a stress response could be associated with the transport of pigs to the abattoir, as well as with the time that the animals are kept in the lairage.

Studies conducted in this thesis investigated porcine granulosa cells, oocytes and/or antral fluids isolated from abattoir-derived ovaries. It is possible that the pigs from which these ovaries were collected could have experienced a period of acute stress during their housing and transportation prior to slaughter. After several hours, exposure to stress may have upregulated cortisol production or induced other endocrine changes which could potentially have affected the expression of the 11βHSD enzymes. The possible implications of a stress response on the endocrine system will now be discussed in further detail.

A rapid but short-lived response to stress is the activation of the sympathoadrenal system, resulting in the secretion of the catecholamine hormones adrenaline and noradrenaline from the adrenal medulla into the systemic circulation. Both adrenaline and noradrenaline appear to downregulate 11βHSD2 gene expression in human trophoblasts (Sarkar *et al.*, 2001). In addition, both catecholamines have been shown to increase levels of cAMP via the activation of β-adrenergic receptors (Hjemdahl *et al.*, 1983). As discussed in chapter 6, cAMP was shown to increase the expression of 11βHSD1 and 11βHSD2 (Sun *et al.*, 1998; Gout *et al.*, 2006). Furthermore, via the pentose phosphate pathway, noradrenaline can inhibit

the production of NAPDH, the cofactor that is required to catalyse the 11-KSR activity of 11βHSD1. In adipocytes, noradrenaline decreased intracellular NADPH and the 11-KSR activity of 11βHSD1 was suppressed while the 11β-DH activity of the 11βHSD1 enzyme increased (McCormick *et al.*, 2006). Vasopressin is another hormone that has been associated with the stress response. This peptide hormone was shown to increase 11βHSD2 activity in isolated rat renal collecting ducts via the vasopressin V2 receptor (and hence presumably via cAMP) (Alfaidy *et al.*, 1997).

Stress can also induce the secretion of the peptide hormone prolactin though there are no reported effects of prolactin on 11βHSD enzyme expression. Furthermore, despite the possible increase of prolactin levels in response to stress, glucocorticoids were shown to inhibit prolactin expression (Whorwood *et al.*, 1993). The effects of glucocorticoids on prolactin production were subsequently shown to be mediated via the GR after the synthetic GR agonist RU 28362 decreased prolactin mRNA levels and prolactin secretion from rat pituitary tumour GH3 cells (Stewart and Whorwood, 1994).

A response to chronic stress, which occurs over several hours, is the activation of the HPA axis and the upregulation of cortisol production (as described in section 1.3.2). It is possible that if the sows experienced a period of stress prior to slaughter at the abattoir, and this resulted in increased plasma cortisol levels, then this may explain the relatively constant cortisol:cortisone ratios in the antral fluids aspirated from abattoir-derived ovaries. Interestingly, as mentioned in section 1.3.3, glucocorticoids appear to protect cells from apoptosis. This was demonstrated in human granulosa cells incubated with dexamethasone, in which the expression of the Bcl-2 proteins was altered (Sasson *et al.*, 2001; Sasson and Amsterdam, 2002). If the sows experienced stressful conditions at the abattoir, this might be expected to activate the HPA axis and ultimately increase physiological cortisol production. According to the findings of Sasson *et al.* (2001) this could protect the cells from undergoing apoptosis subsequent to a

stress response and this anti-apoptotic effect may persist at the time of granulosa cell isolation from the porcine ovaries.

In addition, glucocorticoids have been shown to upregulate β-adrenergic receptor expression and increase cAMP production in cultured human lung cells (Fraser and Venter, 1980). Thus cortisol may indirectly increase the expression of the 11βHSD enzymes via cAMP. The glucocorticoid hormones also appear to affect the expression of both 11βHSD1 and 11βHSD2 directly. Cortisol itself was shown to increase 11βHSD1 expression in pre-adipocytes (Bujalska *et al.*, 1997) and dexamethasone appears to upregulate both the activity and/or expression of both enzyme isoforms in a variety of tissues (Low *et al.*, 1994b; Jamieson *et al.*, 1995; Gao *et al.*, 1997). Therefore, while the 11βHSD enzymes are responsible for metabolising cortisol, the activity and expression of these enzymes could, in turn, be affected by elevated glucocorticoid concentrations, if the animals were stressed prior to slaughter and the collection of tissues. It is possible that, prior to slaughter, the sows did experience acute stress however, a prolonged period of stress over a duration of several hours is likely to be required to affect levels of 11βHSD protein and moreover, enzyme function. Furthermore, as discussed in section 1.3.2, when physiological cortisol concentrations reach adequate (or excessive) levels after a stress response, the glucocorticoid then exerts negative feedback on the HPA axis. Thus, the potential endocrine effects on 11βHSD expression would be alleviated from this point.

Now turning to the results presented in this thesis, in the RT-PCR studies conducted in chapter 3 of this thesis, both 11βHSD1 and 11βHSD2 were shown to be co-expressed in porcine granulosa cells. Co-expression of 11βHSD1 and 11βHSD2 also occurs in the follicular granulosa cells of bovine antral follicles (Tetsuka *et al.*, 2003; Thurston *et al.*, 2007), though only 11βHSD2 is expressed in rat and human follicular granulosa cells (Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Ricketts *et al.*, 1998; Tetsuka *et al.*, 1999a; Tetsuka *et al.*, 1999b). In this thesis, RT-PCR studies conducted in porcine COCs were inconclusive, though preliminary western blotting studies carried out by our research group have

indicated that 11βHSD1 protein is expressed in porcine COCs. 11βHSD1 expression has previously been demonstrated in rat and human oocytes (Benediktsson *et al.*, 1992; Ricketts *et al.*, 1998; Smith *et al.*, 2000). Further work that could be proposed would be to investigate 11βHSD enzyme expression in other cells of the porcine ovarian follicle, such as the theca cells.

Subsequent to the determination of 11βHSD isoform expression in porcine granulosa cells and COCs, the net activities of the 11βHSD enzymes in these cells were measured. No 11-ketosteroid reductase (11-KSR) activities (cortisol regeneration) were displayed in porcine granulosa cells or COCs, irrespective of follicle origin, whereas 11β-dehydrogenase (11β-DH) activities (cortisol oxidation) could be detected in all granulosa cells and COCs. The predominant dehydrogenase activities of 11βHSD1 in steroidogenic gonadal cells have recently been documented (Gao *et al.*, 1997; Michael *et al.*, 1997; Ge and Hardy, 2000; Yong *et al.*, 2000; Tetsuka *et al.*, 2003; Ge *et al.*, 2005; Thurston *et al.*, 2007). Therefore in porcine granulosa cells and COCs, as was suggested to occur in other gonadal cells, the NADPH generated by hexose-6-phosphate dehydrogenase (H6PDH) might be preferentially utilised by the cytochrome P450 (CYP) enzymes in the lumen of the SER. Thus the CYP enzymes would convert NADPH to $NADP^+$, providing an intralumenal supply of $NADP^+$ to drive the 11β-DH activity of 11βHSD1 (Michael *et al.*, 2003; Ge *et al.*, 2005).

Our research group has previously reported that ovarian fluids from porcine large antral follicles and ovarian cysts each contain intrafollicular hydrophobic inhibitors of the activities of 11βHSD1, but not 11βHSD2 (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). In these published studies, the effects of intrafollicular enzyme inhibitors were tested against rat kidney homogenates as a source of both cloned 11βHSD enzymes. The data presented in chapter 4 of this thesis showed that FF from small and medium porcine antral follicles could also suppress 11β-DH activities in renal homogenates. Thus the ovarian fluid from porcine antral follicles at any stage of folliculogenesis has the ability to suppress 11βHSD activities. Porcine cyst fluid consistently exerted a greater inhibition of 11β-DH

activities in rat kidney homogenates, porcine granulosa cells and porcine COCs than did FF from large antral follicles. This suggested that the rates of synthesis and/or metabolism of these intrafollicular enzyme modulators decreased during antral follicle growth, but were increased in ovarian cysts. The high levels of enzyme inhibitors in ovarian cysts could be linked to the occurrence of COD. In the studies of this thesis, and in previously published reports, the endogenous 11βHSD1 inhibitors in porcine ovarian fluids could suppress enzyme activities in renal homogenates during a 1-hour incubation period (Thurston *et al.*, 2002; Thurston *et al.*, 2003b). The short period of time in which these intrafollicular enzyme modulators could act suggested post-translational inhibitory effects on the 11βHSD1 enzyme rather than effects on gene transcription, which would take several hours to occur.

There is evidence to suggest that the changes occurring in intracellular cortisol metabolism within porcine granulosa cells and COCs could be dependent on the levels of the intrafollicular 11βHSD1 inhibitors in the antrum (Figure 7.1), as discussed in chapter 5. It was proposed that significantly decreased levels of intracellular cortisol metabolism may occur in small ovarian follicles to maintain higher levels of active cortisol in the mural granulosa cells and COCs of immature porcine follicles (Figure 7.2). Increasing levels of intracellular glucocorticoid metabolism are associated with antral follicle growth, decreasing the availability of active glucocorticoids in the mural granulosa cells and COCs of preovulatory porcine follicles (Figure 7.3). However, it is apparent that significantly decreased cortisol metabolism in the mural granulosa cells of ovarian cysts can also be associated with the formation of spontaneous ovarian cysts (Figure 7.2). The potential implications of changing exposure to intracellular cortisol on folliculogenesis and oocyte maturation are depicted in Figures 7.2 and 7.3 for each type of follicular structure. The possible effects of the glucocorticoids will also be discussed below.

Firstly, glucocorticoids have been shown to induce differentiation of granulosa cells (Schoonmaker and Erickson, 1983). Therefore in small antral follicles,

intracellular cortisol levels may be increased to promote folliculogenesis by differentiating the various granulosa cell layers in the follicle wall. Preovulatory follicles on the other hand, are in the final stages of folliculogenesis thus intracellular glucocorticoids may possibly be decreased due to a lesser requirement for granulosa cell differentiation in large antral follicles.

Positive effects of the glucocorticoid hormones have been documented on the expression of the connexin proteins Cx26 and Cx32 in rat hepatocytes (Kwiatkowski *et al.*, 1994) and on Cx43 expression in granulosa cells (Sasson and Amsterdam, 2002). All of these connexins comprise the gap junctions between granulosa cells in ovarian follicles (Kidder and Mhawi, 2002). High intracellular cortisol concentrations in small antral follicles and ovarian cysts may thus increase connexin expression in the granulosa cells of each follicular structure. This could possibly aid the formation of the cell communication network in immature antral follicles, thereby potentially driving folliculogenesis. In the case of ovarian cysts however, the upregulation of connexin expression could strengthen the intracellular communication network between the granulosa cells in cystic to prevent cyst degeneration and maintain the phenotype of COD. Conversely in the cells of large antral follicles, low concentrations of cortisol would be predicted to decrease the positive effects of the glucocorticoid on connexin expression. This may destabilise the gap junction communication network between the preovulatory follicle granulosa cells and thus aid the process of follicular rupture prior to ovulation.

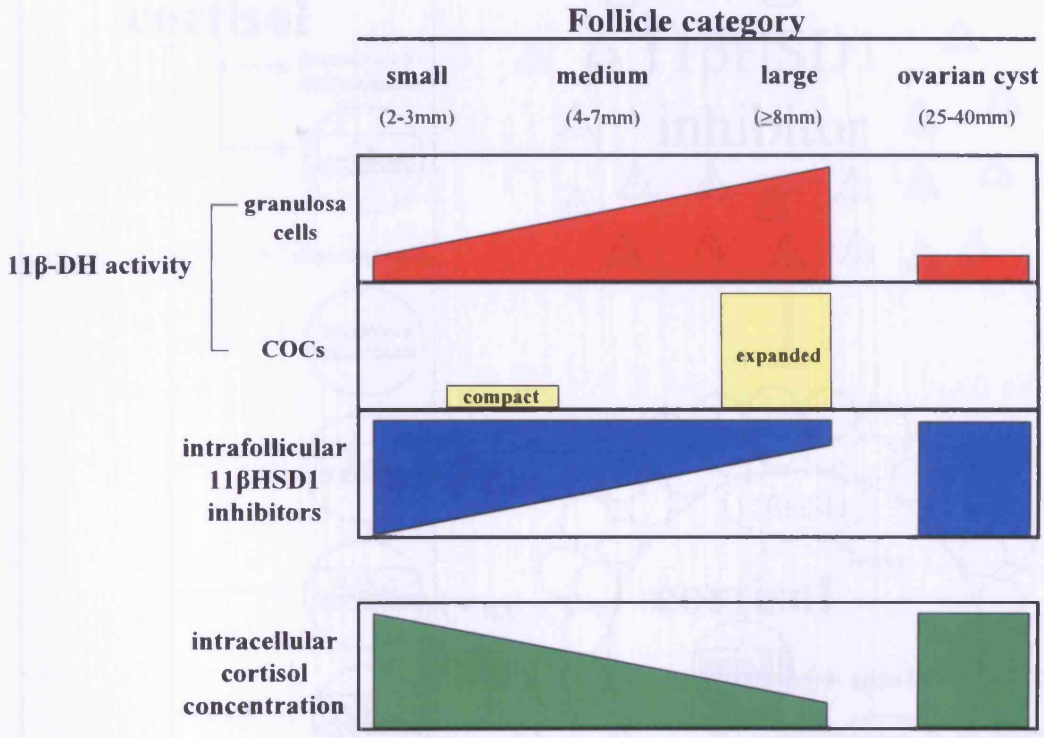


Figure 7.1. The predicted levels of intracellular cortisol (shown in green) in the granulosa cells and COCs of porcine small, medium and large antral follicles or porcine spontaneous ovarian cysts. The red blocks indicate increasing intracellular 11β-dehydrogenase (11β-DH) activities in the granulosa cells with porcine antral follicle growth, but decreased 11β-DH activities in the granulosa cells of ovarian cysts. The yellow blocks represent low 11β-DH activities in compact COCs isolated from small and medium antral follicles but high 11β-DH activities in expanded COCs. The blue blocks indicate decreasing levels of intrafollicular 11βHSD1 inhibitors in FF with porcine antral follicle growth but increased levels of enzyme inhibitors in the fluid of ovarian cysts.

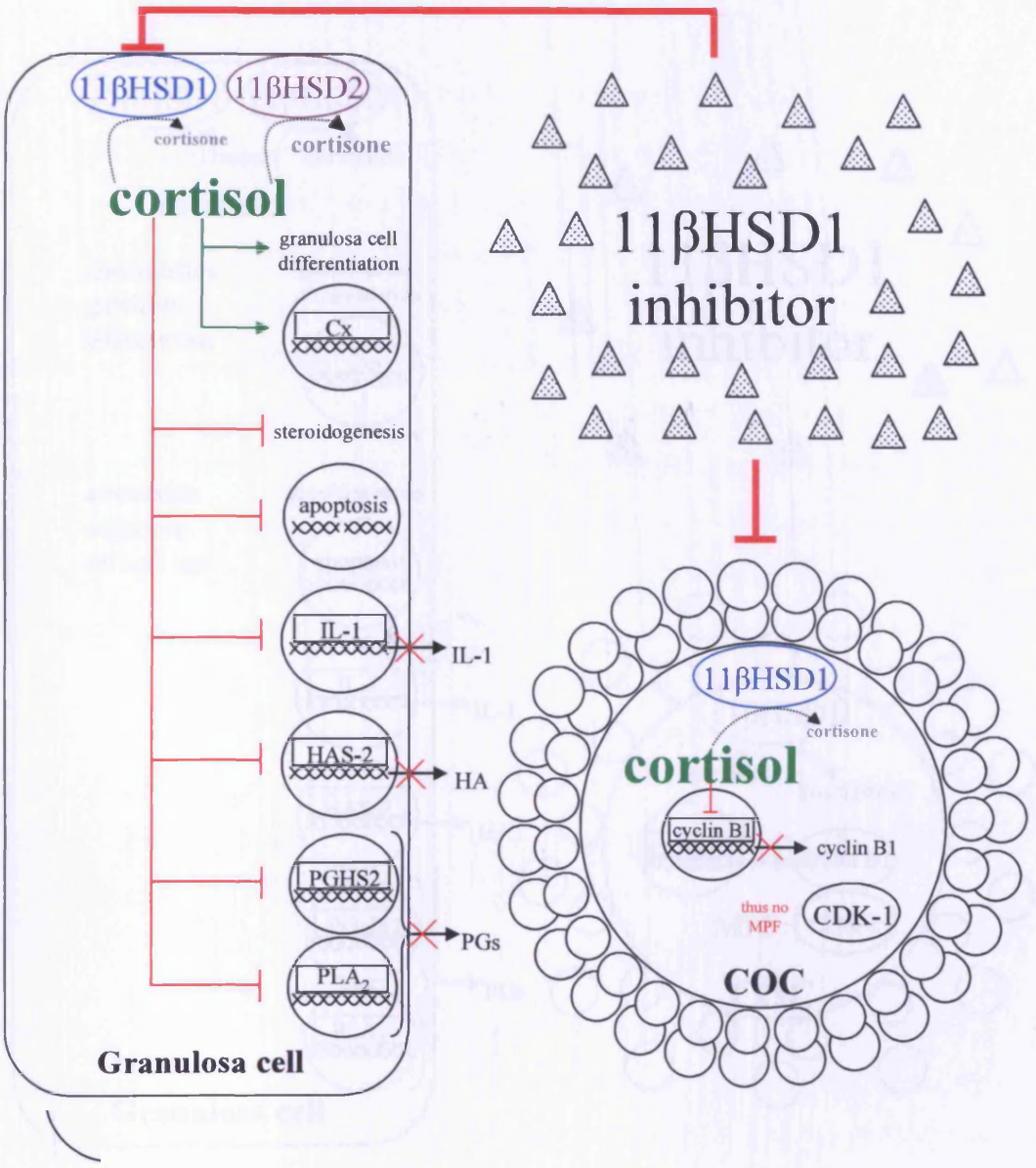


Figure 7.2. Potential consequences of lower levels of intracellular cortisol metabolism in the granulosa cells and COCs of porcine small antral follicles or ovarian cysts. Higher intracellular cortisol concentrations in the granulosa cells may promote granulosa cell differentiation and connexin (Cx) expression, and suppress LH-stimulated steroidogenesis and apoptosis. In addition, cortisol may decrease the transcription of the genes encoding IL-1, HAS-2 (to decrease HA production), and PGHS2 and PLA2 (to limit PG synthesis). Higher cortisol concentrations in the COC may suppress cyclin B1 transcription hence no MPF expression would be expected to occur in the oocyte. In the figure, solid lines depict intracellular effects and dotted lines represent metabolic reactions. The green arrows indicate stimulatory effects of cortisol whereas the red lines suggest inhibitory effects of cortisol.

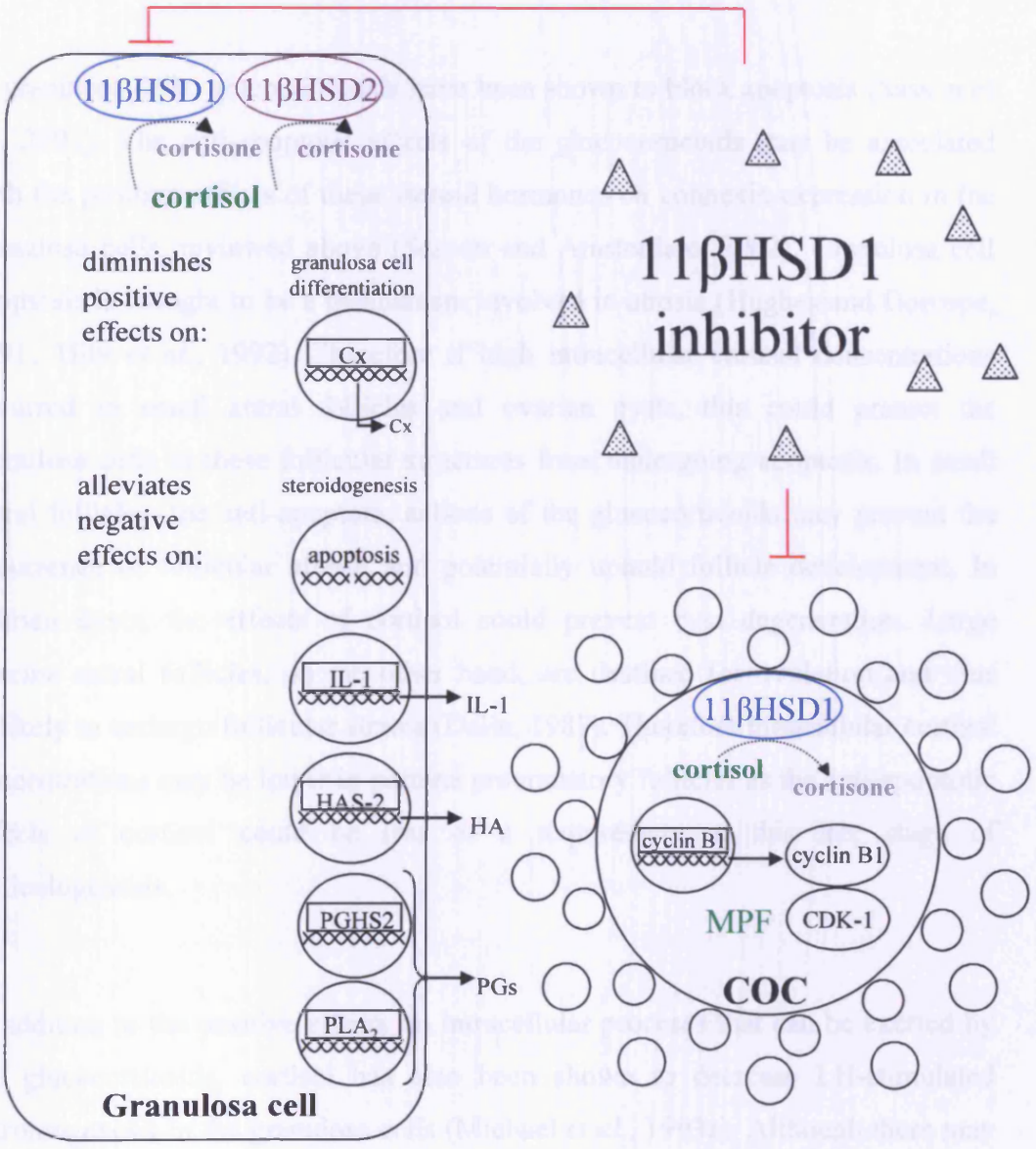


Figure 7.3. Potential consequences of higher levels of intracellular cortisol metabolism in the granulosa cells and COCs of porcine large antral follicles. Lower intracellular cortisol concentrations in the granulosa cells may decrease granulosa cell differentiation and connexin (Cx) expression, and alleviate the suppressive effects of cortisol on LH-stimulated steroidogenesis and apoptosis. In addition, cortisol may support the transcription of the genes encoding IL-1, HAS-2 (to raise HA production), PGHS2 and PLA2 (to increase PG synthesis). Lower cortisol concentrations in the COC may promote cyclin B1 transcription to form MPF in the oocyte. In the figure, solid lines depict intracellular effects and dotted lines represent metabolic reactions.

In granulosa cells, glucocorticoids have been shown to block apoptosis (Sasson *et al.*, 2001). The anti-apoptotic effects of the glucocorticoids may be associated with the positive effects of these steroid hormones on connexin expression in the granulosa cells, reviewed above (Sasson and Amsterdam, 2002). Granulosa cell apoptosis is thought to be a mechanism involved in atresia (Hughes and Gorospe, 1991; Tilly *et al.*, 1992). Therefore if high intracellular cortisol concentrations occurred in small antral follicles and ovarian cysts, this could protect the granulosa cells in these follicular structures from undergoing apoptosis. In small antral follicles, the anti-apoptotic actions of the glucocorticoids may prevent the occurrence of follicular atresia and potentially uphold follicle development. In ovarian cysts, the effects of cortisol could prevent cyst degeneration. Large porcine antral follicles, on the other hand, are destined for ovulation and thus unlikely to undergo follicular atresia (Dalin, 1987). Therefore intracellular cortisol concentrations may be lower in porcine prevoulatory follicles as the anti-apoptotic effects of cortisol could be less of a requirement at this late stage of folliculogenesis.

In addition to the positive effects on intracellular proceses that can be exerted by the glucocorticoids, cortisol has also been shown to decrease LH-stimulated steroidogenesis in the granulosa cells (Michael *et al.*, 1993a). Although there may be high cortisol concentrations in the cells of small porcine antral follicles, these early antral follicles are not likely to be fully LH-responsive. Therefore, high cortisol concentrations may not have a bearing on the steroidogenic output of the cells in these immature follicles. Signs of altered steroidogenesis have been documented in ovarian cysts (Jana *et al.*, 2005) which could be linked to the potentially high intracellular cortisol concentrations in cysts, given that cortisol has the ability to suppress LH-induced ovarian steroidogenesis (Michael *et al.*, 1993a). As an aside, if cortisol has the potential to inhibit steroidogenesis stimulated by LH, then cortisol may suppress the ability of LH to induce other intracellular processes in the follicle, such as cumulus expansion and the induction of ovulation. Thus an ovarian cyst may be anovulatory due to the fact that it was unable to respond to a preovulatory LH surge *in vivo*, given that it could contain

high intracellular cortisol concentration. Large antral follicle cells, on the other hand are expected to be highly dependent on LH-stimulation (see review by Hiller (2001)). Therefore low intracellular cortisol concentrations in granulosa cells of large antral follicles could allow LH-stimulated steroidogenesis to occur and, moreover, could ensure that these follicles can remain fully LH-responsive.

Glucocorticoids have also been shown to decrease intraovarian synthesis of prostaglandins (Goppelt-Struebe, 1997) and pro-inflammatory cytokines, such as interleukin-1 (IL-1) (Telleria *et al.*, 1998). Glucocorticoids were also reported to suppress transcription of hyaluronan synthase-2 (HAS-2) in cultured synoviocytes (Stuhlmeier and Pollaschek, 2004). Prostaglandins, pro-inflammatory cytokines and hyaluronic acid (HA) have all been associated with the occurrence of cumulus expansion and ovulation (see review by Richards (2005)). Therefore, in porcine small antral follicles, high intracellular levels of cortisol may suppress the production of these factors to potentially block the occurrence of ovulation, which would be inappropriate at this stage of folliculogenesis. In the cells of ovarian cysts, the high intracellular cortisol concentrations may have also suppressed the synthesis of prostaglandins, pro-inflammatory cytokines and HA such that cumulus expansion and ovulation may have been prevented in these cystic, anovulatory follicles. This could partially explain the anovulatory status of ovarian cysts. In porcine large antral follicles however, low intracellular cortisol concentrations could be expected to alleviate the potentially negative effects on the synthesis of prostaglandins, cytokines and HA, thus enabling cumulus expansion and ovulation to occur.

Finally, glucocorticoids have been reported to decrease porcine oocyte maturation (Yang *et al.*, 1999) apparently via suppressing the synthesis of cyclin B1 (Chen *et al.*, 2000), a key component of maturation promoting factor (MPF). In porcine small antral follicles and ovarian cysts, high intracellular cortisol concentrations in the COC would be expected to inhibit oocyte maturation. In the case of small antral follicles, the occurrence of oocyte maturation would appear to be inappropriate at this early stage of antral follicle growth. The anovulatory status of

ovarian cysts, on the other hand, could be linked to the suppression of oocyte maturation by high intracellular cortisol concentrations. In large antral follicles however, the increased inactivation of intracellular cortisol within the COC could allow the synthesis of cyclin B1. Thus, in porcine preovulatory follicles, MPF expression could be expected to occur, thus inducing oocyte maturation.

In order to determine whether the glucocorticoid metabolism by the 11βHSD enzymes in ovarian follicle cells could play a role in folliculogenesis, porcine ovarian follicles could be cultured with increasing concentrations of cortisol or dexamethasone. Whereas cortisol can be metabolised by the 11βHSD enzymes dexamethasone cannot, therefore one might expect any observed effects of dexamethasone to appear greater than those of cortisol, if cortisol action was subject to modulation by the 11β-DH activities of 11βHSD enzymes in the cell. At various stages of follicle growth, the granulosa cells could be harvested and effects of either glucocorticoid could be determined on apoptosis, or connexin, IL-1 or HA synthesis in the granulosa cells. Effects on apoptosis could be determined by measuring levels of caspase-9 expression, which was reported to be a mediator of apoptosis in porcine granulosa cells during follicular atresia (Matsui *et al.*, 2003). Additionally, levels of cyclin B1 expression could be assessed in the oocyte during follicle growth.

Cortisone alone could also be cultured with porcine ovarian follicles. Given that no 11-KSR activities were displayed in porcine granulosa cells or COCs, cortisone would not be expected to be reduced to cortisol, thus no effects on follicle growth would be anticipated. If cortisone did exert any effects on folliculogenesis however, this would raise the possibility that the 11βHSD1 enzyme in the cells of intact porcine follicles have the potential to display 11-KSR activities, enabling the local regeneration of cortisol to affect follicle growth. In this case, follicles could be co-treated with cortisone plus a specific inhibitor of the 11-KSR activity of 11βHSD1 to see if the effects of cortisone could be abolished by inhibiting the ability of 11βHSD1 to reduce cortisone to cortisol.

Additionally, a role for the 11βHSD enzymes during follicle growth could be determined by inhibiting 11βHSD activities in the cells of cultured porcine ovarian follicles. Recently, reports of a number of pharmacological inhibitors of 11βHSD1 have been published, including the BVT compounds, the first of which to be described was BVT.2733 (Alberts *et al.*, 2002). Alternatively, 11βHSD expression in the ovarian follicle cells could be knocked down or knocked out using siRNA or MAOs, as dicussed in chapter 6. If cortisol action was potentiated by impairing the activity of the 11βHSD enzymes in the ovarian follicle cells then this would indicate a role for the enzymes in metabolising cortisol during follicle growth. Similar studies were proposed in chapter 6 to determine the effects of suppressing 11βHSD activities in porcine oocytes on the rates of maturation.

Another focus for further work would be the investigation of potential roles for the GR and MR in folliculogenesis and oocyte maturation. Expression of both the GR and MR has been shown to occur in the granulosa cells and the oocyte (Tetsuka *et al.*, 1999b; Robert *et al.*, 2000) and both of these receptors can be activated by cortisol. The findings of the study by Tetsuka *et al.* (1999b) suggested that levels of GR expression do not change during preovulatory follicle development whereas MR expression was shown to decrease with follicle growth. Thus there may be a role for MR expression during folliculogenesis and potentially in oocyte development. Based on the findings of Tetsuka *et al.* (1999b), high levels of MR expression would be anticipated in the cells of immature ovarian follicles. In small porcine antral follicles levels of intracellular cortisol metabolism would appear to be low, both in the mural granulosa cells and in the immature COCs. This could potentially increase the exposure of MR in the cells of small antral follicles to promiscuous activation by the glucocorticoid steroid, cortisol. Hence during the early stages of antral follicle growth, cortisol may exert effects inside the granulosa cells via activation of the MR as well as the GR.

It would be interesting to ascertain whether GR or MR expression occurred in the cells of porcine ovarian follicles and furthermore, whether levels of expression

changed in ovarian follicle cells during follicle growth. One could also investigate whether the action of either receptor was involved in folliculogenesis or oocyte maturation. To examine the role of the GR or the MR, follicles or oocytes could be incubated with RU-486 (a pharmacological GR antagonist) or spironolactone (an antagonist of MR) to determine potential effects of the loss of receptor stimulation on follicle growth and/or oocyte development. Alternatively GR and/or MR expression could be knocked down or knocked out using siRNA or MAOs to ascertain whether folliculogenesis and/or oocyte maturation could occur in the absence of GR or MR activation. If the potential effects of cortisol on folliculogenesis and/or oocyte development were blocked by the addition of RU-486 or in the absence of GR expression then it would appear that cortisol was exerting effects through the GR. Since cortisol can also activate the MR it might also be worth to determine any effects of cortisol on folliculogenesis and/or oocyte maturation in the presence of spironolactone. If any effects of cortisol were abolised then this could indicate that cortisol was affecting follicle growth or oocyte development through the activation of the MR.

Turning to the identity of the intrafollicular enzyme inhibitors, Thurston *et al.* (2003b) first suggested that progesterone was not likely to be the main enzyme inhibitor, given that progesterone could inhibit 11βHSD2 whereas the enzyme inhibitors in the ovarian fluids could not. In the study presented in chapter 4 of this thesis, further evidence was provided to potentially discount progesterone as the main endogenous enzyme inhibitor in antral fluid. Though the identity of the main intrafollicular 11βHSD1 inhibitor is still unknown, it appears that a wide range of hydrophobic compounds in porcine ovarian fluids can suppress $NADP^+$-dependent 11β-DH activities in rat kidney homogenates. This raises the possibility that the endogenous 11βHSD1 inhibitors in porcine ovarian fluids could be steroids or sterols derived from cholesterol, with varying degrees of hydrophobicity as indicated by their chemical structures. A number of cholesterol derivatives have been reported to be metabolised by 11βHSD1, or to inhibit the activity of the enzyme. These include the metabolites of dehydroepiandrosterone (DHEA), oxysterols and the bile acids (Perschel *et al.*, 1991; Latif *et al.*, 1994; Gu *et al.*, 2003; Robinzon *et al.*, 2003; Hult *et al.*, 2004; Schweizer *et al.*, 2004;

Apostolova *et al.*, 2005; Robinzon and Prough, 2005; Muller *et al.*, 2006). Given the hydrophobic nature of the intrafollicular enzyme inhibitors, GC-MS could be employed to identify the chemical nature and/or identity of the inhibitory compounds in porcine ovarian fluids.

As well as ascertaining the identity of the endogenous 11βHSD inhibitors, their site of origin would also need to be investigated. These compounds may be synthesised at an external site of production and thus enter ovarian follicles within the serum transudate. Examples of compounds thought to enter follicle via the systemic circulation are bilirubin and beta-carotene, which give FF its yellow appearance (Bayer *et al.*, 1990; Bayer *et al.*, 1992). Alternatively, the compounds which modulate 11βHSD activity could be synthesised by the ovarian follicle cells and then secreted into the antral fluid. Indeed studies have documented evidence of cholesterol metabolism in ovarian homogenates, as well as in specific cells of the ovary (Bjorkhem and Danielsson, 1974; Longcope, 1986; Rennert *et al.*, 1990; Su *et al.*, 1990; Axelson *et al.*, 1991; Furster, 1999; Wu *et al.*, 1999).

In summary, the findings of this thesis have indicated that the 11βHSD enzymes are expressed in the granulosa cells and the COC in porcine ovarian follicles. These 11βHSD enzymes display predominant, if not exclusive, 11β-DH activities in granulosa cells, oocytes and COCs. The 11β-DH activities increase in the granulosa cells, oocytes and COCs with antral follicle growth, during which levels of the intrafollicular 11βHSD1 inhibitors decrease in FF. Thus the endogenous enzyme inhibitors could modulate the metabolism of cortisol in ovarian follicles by regulating the dehydrogenase activities of the 11βHSD enzymes in the ovarian follicle cells. The endogenous enzyme inhibitors may additionally be involved in inducing porcine oocyte maturation in preovulatory follicles. Cortisol metabolism appears to be significantly decreased in ovarian cysts, in light of the significantly decreased levels of net cortisol oxidation in the granulosa cells of cysts and the high levels of endogenous enzyme inhibitors in cyst fluid. Thus cortisol metabolism appears to play a role in folliculogenesis, and altered cortisol

metabolism appears to be associated with the formation of spontaneous ovarian cysts.

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